Vol. XLVII March 1958 No. 3

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

This Issue in Two Parts

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Published for the
GENERAL MEDICAL COUNCIL
by
THE PHARMACEUTICAL PRESS, 17 BLOOMSBURY SQUARE, LONDON, W.C.1.
Obtainable from: The Rittenhouse Book Store. 1706 Rittenhouse Square, Philadelphia, 3

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVII | *MARCH 1958* | NUMBER 3

The Separation and Determination of Reserpine, Deserpidine, and Rescinnamine By Partition Column Chromatography*

By ALMA L. HAYDEN, LEONARD A. FORD, and ALBERT E. H. HOUK

A method is proposed for the column chromatographic separation of deserpidine, reserpine, and rescinnamine, and their determination by ultraviolet spectrophotometry. The method is useful for determining the purity of the crystalline alkaloids, and is suitable for the assay of their commercial mixtures.

IN THE COURSE of investigations in this laboratory on Rauwolfia alkaloids in therapeutic preparations, the need of a method for the quantitative separation of reserpine, deserpidine, and rescinnamine became apparent. This need was heightened by the lack of absolutely pure specimens of the three alkaloids. All available crystalline samples of the three substances were found to contain small amounts of one or both of the other congeners.

Carol and his collaborators (1, 2) have developed a qualitative procedure for separating reserpine, deserpidine, and rescinnamine by paper chromatography, using an isooctane-carbon tetrachloride-piperidine-tertiary butyl alcohol-formamide system. The procedure presented here separates the three alkaloids quantitatively by liquid-liquid partition column chromatography. The alkaloids are then determined by direct ultraviolet spectrophotometry of the eluates in the region between 260 and 330 mμ. The solvent system is *n*-heptane-chloroform-morpholine-formamide. Celite® 545 as obtained is used as the solid supporting material.

EXPERIMENTAL

Materials.—Solvents—(a) U. S. P. chloroform and methanol; redistilled *n*-heptane and morpholine. On comparison with water at 272 mμ, the optical density values of these solvents should be less than 0.200.

(b) Formamide. Distill in all glass apparatus under vacuum. Reject the first portion of distillate containing water, and collect the fraction which boils at about 101°/12 mm. Hg (115°/25 mm. Hg).[1] Store the purified formamide over concentrated sulfuric acid to remove most of the ammonia. The formamide should be neutral or slightly alkaline.

Diatomaceous Silica Support—Celite® 545, produced by the Johns-Manville Co.

Eluent—Mix thoroughly, in a separatory funnel, 715 ml. of *n*-heptane, 110 ml. chloroform, 1 ml. of morpholine, and 25 ml. of formamide. After equilibration (about 20 minutes) separate the formamide layer and retain it for use as the immobile solvent. Filter the eluent (top layer) through $CHCl_3$-washed cotton. Prepare the solvent system on the day of use.

* Received December 26, 1957, from the Division of Pharmaceutical Chemistry, Bureau of Biological and Physical Sciences, FDA, Department of Health, Education, and Welfare, Wash., D. C.

[1] Some commercial formamide contains a strongly absorbing impurity which is not removed completely on distillation under the above conditions. Adsorption chromatography on Fisher alumina removes this impurity sufficiently to allow the direct use of the filtered eluate for spectrophotometry. However, many formamide distillates with optical densities up to 1.3 at 272 mμ, when compared with water in 1 cm. silica cells, were used without apparent effect upon the absorption of the eluates or the resolution of the alkaloids.

Preparation of Standard Solutions of Reserpine, Deserpidine, and Rescinnamine.—Accurately weigh about 1 mg. of each alkaloid. Dissolve each sample in a few drops of chloroform and dilute to 100 ml. with the filtered eluent. Measure the absorbances of these solutions between 260 mμ and 330 mμ in a suitable spectrophotometer relative to the eluent as blank. Since there is some decomposition of these alkaloids on standing in the eluent, the readings should be made immediately after preparation of the solutions. In the concentration range studied (up to 0.020 mg./ml.) solutions of these alkaloids in eluent and eluate follow Beer's law at their respective absorption maxima. To permit direct reading of the eluates in the ultraviolet spectrophotometer, the use of the eluent as reference blank is necessary.

Chromatographic Tube.—A 350 mm. length of glass tubing (i. d. 21 mm.) fused to a piece of tapered outlet tubing.

Preparation of the Column—Place a small pledget of $CHCl_3$-washed cotton in the bottom of the chromatographic tube. Thoroughly mix 25 Gm. of Celite and 20 ml. of the immobile solvent. Add this mixture to the tube through a powder funnel in five equal portions by weight and tamp each portion tightly to a height of 4.8 cm. Add about 0.5 Gm. of Celite to the column and tamp down tightly.

Preparation of the Samples—Dissolve an accurately weighed sample[3] containing 0.5–5.0 mg. of the alkaloid(s) in about 1 ml. of chloroform-methanol (3 + 1) and transfer to a 30-ml. beaker containing 1 Gm. of Celite, with the aid of several small portions of chloroform-methanol. Mix thoroughly and allow the solvent to evaporate under a hood fan or under vacuum at room temperature.

Add the sample-Celite mixture to the prepared column using about 0.5 Gm. of Celite to rinse the sample beaker. Add a small pledget of $CHCl_3$-washed cotton to the top of the column and tamp down tightly. Rinse the sample beaker three times with about 2 ml. of eluent and add the rinsings to the column.

Place the remainder of the eluent in a stoppered separator as reservoir, and allow the eluent to percolate through the column. Adjust the level of liquid above the column to yield an initial flow rate of about 65–70 drops of eluate per minute (5 ml. in three minutes). Develop the chromatogram at 23°–26°.

In general, for amounts up to 1.5 mg. of total alkaloids, collect a 100-ml. forerun; four 5-ml. fractions; deserpidine in the next 75 ml.; five 5-ml. fractions; reserpine in the next 110 ml.; six 10-ml. fractions; rescinnamine in the next 200 ml. and finally five 5-ml. fractions. The fractions separating the alkaloids are collected in 5-ml. portions to establish the minimum absorption before and after the elution of each alkaloid, to permit the detection of any trailing between zones, and to detect early elution of the alkaloids. The time required for complete elution of the alkaloids is about six hours.

For amounts greater than 1.5 mg. collect sufficient 5 ml. fractions (on the basis of ultraviolet readings) before and after each main eluate to detect any early elution and trailing due to the larger amount of sample. For the determination of alkaloids present to the extent of less than 0.50 mg. collect the appropriate fraction in 5-ml. portions of eluate.

Determine the ultraviolet absorption spectra of the various fractions relative to the eluent as blank between 260 mμ and 330 mμ. Make any necessary adjustments in the observed readings for overcompensation of eluent. The eluates containing deserpidine show a maximum absorption at 272 mμ; reserpine shows maxima at 266–268 mμ and 295 mμ; and rescinnamine shows a maximum at 303–305 mμ. Calculate the quantities of alkaloids in the eluates by comparing their absorbances, corrected for background absorbance, with those of the corresponding standard solutions.

[3] Aliquots of alcoholic solution of the alkaloids may be concentrated in vacuo to give the desired amount of sample.

DISCUSSION

The proposed method of analysis was applied to crystalline samples of reserpine, deserpidine, and rescinnamine, and to synthetic and commercial mixtures of the alkaloids. The purest samples of the three alkaloids were chosen as the standards. The elution of the three alkaloids in the order of deserpidine, reserpine, and rescinnamine is consistent with their relative R_f values in paper chromatograms.

The average recoveries of deserpidine, reserpine, and rescinnamine were 99, 97, and 95%, respectively, in the analysis of both individual alkaloids and synthetic mixtures. These recoveries were corrected for the presence of alkaloidal impurities in the standards. One to two mg. of crystalline material was used for the assay of individual alkaloids.

Results of chromatographic analysis by the proposed method are listed in Table I. Recoveries for the reserpine samples are based on direct comparison with the standard solutions. Samples R6 through R10 therefore contain as much pure reserpine as the standard, while samples R1 through R5 are contaminated with appreciable quantities of other alkaloids.

For the detection of very small amounts (1–5%) of one alkaloid in a sample of another, 1–5 mg. of sample was used. Amounts up to 10 mg. of an individual alkaloid can be analyzed for small amounts of the congeners if the concentration of the adjacently eluted congener does not exceed 3%; however, much larger volumes of eluent are required, and considerable trailing between zones occurs. In addition, when these larger amounts are used, the recovery of the main constituent is lowered.

Two per cent rescinnamine was indicated in the deserpidine standard. Both deserpidine (1%) and rescinnamine (1%) were indicated in the reserpine standard. The rescinnamine standard contained 2% reserpine.

Figure 1 shows the complete chromatographic separation of 1.5 mg. of a synthetic mixture containing 33% deserpidine, 33% reserpine, and 34% rescinnamine. With concentrations up to about 0.5 mg. of each component of a mixture there is complete separation. With increasing concentrations of one or more of the components, larger volumes of eluent may be required, and considerable trailing of one fraction into an adjacently eluted fraction may occur. However, complete separation is obtained when increasing concentrations of one component is accompanied by decreasing concentrations of the other component(s) (Samples 3 and 4). Some crys-

TABLE I.—CHROMATOGRAPHIC ANALYSIS OF INDIVIDUAL ALKALOIDS AND MIXTURES

Sample Number	Description	Deserpidine, % Declared or Added	Deserpidine, % Found	Reserpine, % Declared or Added	Reserpine, % Found	Rescinnamine, % Declared or Added	Rescinnamine, % Found
1	0.50 mg. Standard Deserpidine	100	98	..	..	..	2.0
2	1.956 mg. Standard Reserpine	..	0.85	100	96.0	..	1.3
3	1.760 mg. Standard Rescinnamine	..	..	..	2.2	100	94.4
4	Synthetic mixture of 0.098 mg. Deserpidine and 2.7 mg. Reserpine +0.095 mg. Rescinnamine	4.14	4.04	91.8	90.0	4.01	3.9
5	Synthetic mixture of 0.098 mg. Deserpidine +0.122 mg. Reserpine +1.65 mg. Rescinnamine	5.24	5.24	6.52	6.5	88.0	90.0
6	1.0 mg. Commercial mixture M1	25.0	25.5	75.0	73.0	..	..
			24.6		72.3	..	2.3
7	1.1 mg. Commercial mixture M2	20.0	20.2	60.0	59.8	20.0	19.6
8	2.0 mg. Commercial Reserpine R1	0	14.1	100	87.8	0	0.0
9	2.0 mg. Commercial Reserpine R2	0	3.7	100	92.6	0	0.0
10	2.5 mg. Commercial Reserpine R3	0	9.8	100	74.8	0	3.2
11	4.0 mg. Commercial Reserpine R4	0	5.7	100	89.0	0	..
			5.7		90.5		1.1
12	5.1 mg. Commercial Reserpine R5	0	4.2	100	87.2	0	4.7
13	2.0 mg. Commercial Reserpine R6	0	1.2	100	95.7	0	0
14	2.0 mg. Commercial Reserpine R7	0	1.4	100	95.9	0	0
15	2.0 mg. Commercial Reserpine R8	0	0.6	100	96.1	0	0
16	2.0 mg. Commercial Reserpine R9	0	0.5	100	95.9	0	..
17	2.0 mg. Commercial Reserpine R10	0	1.0	100	95.7	0	0.8
18	2.0 mg. Commercial Deserpidine D1	100	95.1	0	2.7	0	0
19	2.0 mg. Commercial Deserpidine D2	100	89.0	0	7.1	0	0.8
20	2.0 mg. Commercial Deserpidine D3	100	93.8	0	3.8	0	0

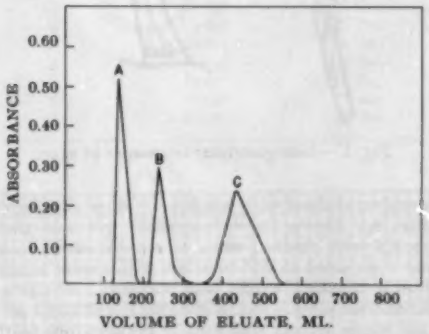

Fig. 1.—Chromatographic Separation of Deserpidine (A), Reserpine (B), and Rescinnamine (C).

talline reserpine samples, containing 1 to 14% deserpidine and 1 to 5% rescinnamine were analyzed in amounts up to 2 mg. with excellent results.

The elution pattern of the alkaloids varies when the experimental conditions are altered. The use of a larger proportion of formamide and/or the omission of morpholine in the solvent system results in lower recoveries of the alkaloids. A larger proportion of chloroform results in earlier elution and poor separation of the alkaloids. Increase or decrease in the amount of Celite alters the volumes of eluent required, the time of elution, and the resolution of the alkaloids. An increase in flow-rate decreases the resolution and the time of elution.

Under the given experimental conditions the volume of eluent required and the position of each alkaloid in the elution pattern are reproducible within the optimum concentration ranges. When these conditions do not exist, it is necessary to track the chromatogram by analysis of smaller cuts.

Table II presents a comparison of results obtained for crystalline reserpines by column chromatography, by semi-quantitative paper chromatography (1, 2), and by the nitrite procedure (3). The excellent agreement among these independent methods indicates that all of them are useful for the analysis of reserpine preparations. Investigations on the application of the proposed method to the analysis of Rauwolfia root extracts will be reported elsewhere.

TABLE II.—ANALYSIS OF CRYSTALLINE RESERPINE BY VARIOUS METHODS

Sample	Deserpidine, % Paper	Deserpidine, % Proposed Method	% Reserpine Plus Rescinnamine Nitrite[a]	% Reserpine Plus Rescinnamine Proposed Method
R1	Over 8%	14.1	85.0	87.8
R2	About 4%	3.7	93.8	92.6
R3	Over 8%	9.8	77.7	78.0
R4	Over 4%	5.7	94.6	91.6
R5	About 4%	4.2	..	91.9
R6	Less than 2%	1.2	..	95.7
R7	Less than 2%	1.4	..	95.9
R8	Less than 2%	0.6	98.8	96.1
R9	Less than 2%	0.5	97.3	95.9
R10	Less than 2%	1.0	96.3	96.5

[a] This method does not differentiate between reserpine and rescinnamine.

REFERENCES

(1) Banes, D., Carol, J., and Wolff, J., THIS JOURNAL, 44, 640(1955).
(2) Carol, J., private communication.
(3) Banes, D., Wolff, J., Fallscheer, H. O., and Carol, J., THIS JOURNAL, 45, 710(1956).

Trichome Variability in Commercial Sages*

By H. I. SILVERMAN and A. URDANG

A quantitative ratio of the number of trichomes occurring in a definite weighed amount of dried sage leaf has been determined. A mean has been calculated upon which further analyses of sage in foods can be based. Also, quantitative measurements of the basal cells of seven samples of sage have been tabulated.

IN A QUANTITATIVE microanalytical study recently undertaken (1), it was noted that the nonglandular uniseriate hairs of Dalmatian sage varied in number from lot to lot in samples studied. Accordingly several batches of *Salvia officinalis* Linn. occurring on the market were purchased and microscopically analyzed for number of trichomes per weight of dried leaf.

It was thought that a permanent record (2) of the variation and number of trichomes per weight of dried leaf, or drug, would be valuable for microanalytical and/or quantitative plant work.

Microscopically the leaf of *Salvia officinalis* Linn. is covered on both its upper and lower epidermis with nonglandular uniseriate hairs, one- to five-celled and generally curved. The end cell is attenuated and the basal cell thicker in its walls with a narrower lumen (Fig. 1). Upon comminution of sage leaves the hairs are broken up into varying lengths; however, the basal cells remain intact, probably due to the greater thickness and consequently increased strength of their walls as compared to the other trichome cells.

After extended investigation it was found that these basal cells, in a ground sample, could be utilized for a counting procedure in which the number of trichomes per weight of comminuted leaf was determined.

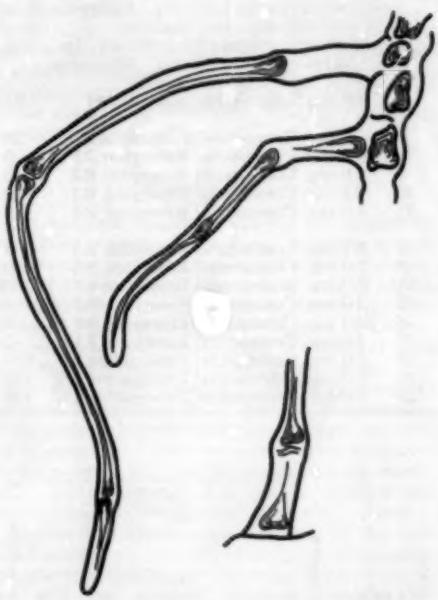

Fig. 1.—Non-glandular trichomes of sage.

METHOD

The samples were first identified by suitable macro and microscopical methods and their commercial designations verified. A representative sample of each of these purchased samples was then ground sufficiently fine to pass entirely through a number 80-mesh sieve and then treated as follows in order to aid the microscopist in his recognition of the basal cells during the counting procedure.

The ground sage was treated with ether (at the boiling point) to remove interfering pigments, fats, and oils. After removal of the ether, the sample was then stained with a 0.5% aqueous solution of Bismarck brown for five minutes. It was then washed with distilled water to remove excess stain and oven dried at 50° (not less than twelve hours) until a constant weight was obtained. The powdered dried sample was thoroughly mixed and a portion whose weight was usually between one-tenth and one milligram was removed and accurately weighed on a microchemical balance. After weighing, the material was carefully transferred to a lucite plate measuring 6 x 5 cm. with a 4-cm. square etched net-like ruling of 1-mm. spaced lines (Fig. 2). A few drops of a 2% sodium carboxymethylcellulose[1] gel were placed on the plate, and mixed with the sample, and the plate was then covered with a suitable cover glass. Examination and counting of the basal cells observed was carried out in the usual manner using the etched lines of the lucite plate as an aid in scanning the sample covered area.

A minimum of four separate portions were removed from each of the stained samples and counted.

DATA

Table I illustrates the weights taken of each sample, hairs counted, and hair indices (number of

* Received May 20, 1957, from Long Island University, Brooklyn College of Pharmacy, N. Y.

[1] CMC-70-D High, Hercules Powder Co.

TABLE I.—TRICHOME COUNT OF SAGE LEAVES

Sample No.						Av. No. Hairs/ mg.
1	mg.	0.456	0.368	0.447	0.490	...
	Hairs	349	225	271	254	625
	Index	765	611	606	518	...
2	mg.	0.461	0.466	0.265	0.378	...
	Hairs	322	313	151	189	610
	Index	698	672	570	500	...
3	mg.	0.549	0.483	0.312	0.928	...
	Hairs	349	298	352	528	599
	Index	636	617	575	569	...
4	mg.	0.489	0.662	0.529	0.841	...
	Hairs	250	296	236	370	461
	Index	511	447	446	440	...
5	mg.	0.569	1.026	0.709	0.937	...
	Hairs	323	463	300	346	453
	Index	568	451	423	369	...
6	mg.	1.252	1.113	1.041	0.739	...
	Hairs	578	476	428	270	417
	Index	462	428	411	365	...
7	mg.	0.186	0.427	0.537	0.766	...
	Hairs	71	152	191	184	334
	Index	382	356	356	240	...

hairs per mg. of dried leaf) and their averages. Table II indicates some notable characteristics of each of the samples analyzed. Quality of drug was derived after determining volatile oil content, foreign organic matter, and filth contained in each lot (3, 4). Also in Table II are microscopical measurements (in microns) of the basal cells. Each number represents an average of twenty determinations on as many trichomes.

DISCUSSION AND CONCLUSION

As can be noted in Tables I and II, the poorer samples have a lower hair count when compared to choicer lots of drug. If a new lot of sage were ground and four samples taken, the mean hair count for these four samples would be 500 ± 102 (5). If the count were 398 or lower, or 602 or higher, a 95% certainty would then exist that the new lot differs from the above standard mean.

Hair counts obtained by the manner described can be utilized in making percentage determinations of sage leaf in various spiced foods, as will be shown in a subsequent paper, by simply extracting the vegetable material and analyzing for hair content. It is necessary, however, to take into consideration that a considerable variation may occur from lot to lot due to fertility of soil, climate, length of growing season, etc. The hair count thus obtained will indicate the content of sage leaf by comparing with suitable charts of hair count on pure samples of commerical sage.

TABLE II

Sample No.	Quality	Amount Purchased	Entire or Powdered Leaves	Basal Cell Dimensions, μ Width	Length
1	Good	2 lbs.	Powdered	11.4	12.9
2	Good	2 lbs.	Entire	10.9	13.2
3	Good	4 oz.	Powdered	11.2	14.0
4	Good	2 lbs.	Entire	11.1	10.5
5	Poor	1 lb.	Entire	11.3	13.3
6	Poor	4 oz.	Entire	11.0	12.0
7	Poor	2 lbs.	Entire	17.5	21.0

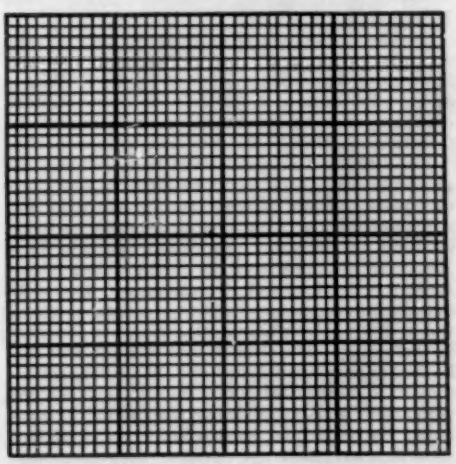

Fig. 2.—Lucite plate ruling.

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A Study of the Root of *Morinda citrifolia* Linné I*

By HEBER W. YOUNGKEN, Sr.†

Morinda citrifolia Linné, a shrub or small tree of the *Rubiaceae* family native to southern Asia, the Philippines, and islands of the South Pacific, possesses roots, extracts of which have been stated to possess hypotensive and anticongestive properties, but very scanty work has been recorded on their pharmacognosy. In this paper the physical characteristics, histology, and description of the powdered root are discussed. Among the diagnostic anatomical characteristics of this drug are the following: the presence of raphides of calcium oxalate in raphide sacs of the cortex and phloem; a banded secondary xylem; vessels with closely set, circular bordered pits; abundant starch-bearing wood parenchyma; vascular rays from 1- to 5-cells in width; stone cells in both cortex and phellem; starch grains, simple and 2- to 3-compound. The presence of an endotrophic fungus with septated, branched hyphae was observed in all tissues of the roots obtained from Luzon.

THIS STUDY WAS PROMPTED by the report in the literature by Dr. Dang Van Ho of Viet Nam (1) who stated that the total extract of the root of *Morinda citrifolia* L. was effective in 81 per cent of his 58 patients suffering from hypertension without having observed a single instance of intolerance and, also, that the drug was shown to possess anticongestive properties in the treatment of hemorrhoids, nasal congestion, and cerebral hemorrhage.

Most of the prior investigations on this drug have been concerned with chemical constituents and microchemic tests and with the root bark and wood, the former having been used in India for many years as a source of a dye. In 1848, Anderson (2) isolated from the root bark of *M. citrifolia* a crystalline substance possessing the formula $C_{28}H_{30}O_{15}$ and named it morindin. Later, Oesterle (3) isolated from this bark a substance, which he found to be trioxymethylanthraquinone monomethyl ether to which he ascribed the formula ($C_{16}H_{10}O_5$). Oesterle and Tisza (4) isolated two other anthraquinone substances which they named morindadiol ($C_{15}H_{10}O_4$), m. p. 244°; and soranjidiol ($C_{15}H_{10}O_4$), m. p. 270°; and also a wax ($C_{18}H_{38}O$) in white needles, m. p. 124.5°. In 1908, Tunmann (5) published a brief description of the wood and root and, by means of microchemic tests, found morindin in the medullary rays, soranjidiol in the phloem parenchyma, and morindadiol in the sieve tubes.

Since *M. citrifolia* roots have been imported for screening into the U. S. A. and as there is a possibility of the drug being used here in the future, this investigation was undertaken in order to provide sufficient pharmacognostical characterization for its identification.

* Received May 3, 1957, from the Massachusetts College of Pharmacy, Boston 15, Mass.
Presented to the Scientific Section, A. PH. A., New York meeting, May, 1957.

† Research Professor of Pharmacognosy and Botany, Massachusetts College of Pharmacy.

MATERIALS AND METHODS

The materials used in this study included certified herbarium specimens of leaf and flowering and leaf and fruiting branches of *M. citrifolia* L. Sources[1] of materials were as follows: one, with a portion of the root system attached, collected by Prayob Sinchermeiri in Bangkok, Thailand, in 1956 and furnished the writer by Dr. K. Savatabandahu of the Section of Systematic Botany of Bangkhen Experimental Station, Bangkok; another with roots and a photograph of the plant from which they were collected in Calcutta, India, from Dr. S. C. Datta of the Central Drugs Laboratory, Calcutta; nearly five pounds of roots of different thicknesses of *M. citrifolia* L., donated by Riker Laboratories; a herbarium sheet of leaf and flowering branch and root, gathered by Dr. Ed. Quisumbing in the Philippines; and roots of *M. citrifolia* L., gathered in Luzon and certified by Dr. R. E. Woodson, Jr., Senior Taxonomist at the Missouri Botanical Garden.

The herbarium materials were compared with a certified sheet of *M. citrifolia* L. in the Harvard Herbarium, Cambridge, collected by E. H. Wilson at Kigo, Formosa, in 1918; and with the description of this species by J. Pitard in "LeComte Flora Generale Indo China" (6), and found to be authentic.

All of the root materials were first examined organoleptically. After recording their physical characteristics, samples of them were prepared for examination by the usual pharmacognostic methods discussed by the writer in previous papers in THIS JOURNAL. Sections were cut by the sliding microtome, and by freehand sectioning, and separately examined in temporary mounts in chloral solution, water, KOH solution, and other reagents discussed in this paper. The slender ends of roots were imbedded in paraffin and sectioned on a rotary microtome. Numerous temporary mounts and stained permament mounts of transverse, radial longitudinal, and tangential longitudinal sections of root specimens were made and examined microscopically. Schulze's maceration process was used in the technique of separating the lignified elements for study. Numerous measurements of tissue elements were made and recorded.

[1] To each of the suppliers of materials used in this study, the author extends his hearty appreciation.

The Plant.—*Morinda citrifolia* L., commonly known as Indian Mulberry and/or Bankoro, is a glabrous shrub or tree of the *Rubiaceae*, attaining a height of 3–10 M. It occurs in India, Indo-China, Malaya, Thailand, Phillippines, and Polynesia. From studies of herbarium specimens, the leaves are broadly elliptic to elliptic-obovate; 10–31 cm. in length; acute, acuminate, or obtuse, less often emarginate, at the summit; cuneiform at the base; the margin nearly entire or undulate; coriaceous in texture; the lamina with from 6 to 7 arcuate, ascending nerves which alternate on each side of the midrib. The petiole measures up to 12 mm. in length. The stipules are interpetiolar, suborbicular, and 2- to 3-lobed at their summit. The stem branches are quadrangular and jointed. The inflorescence consists of solitary axillary capitula of ovate to spheroidal shape, bearing tubular white flowers. The flowers show a gamosepalous calyx with a truncate limb; a 5-lobed, tubular white corolla; 5 stamens with short, hairy filaments; and a pistil with a 2-celled ovary. The fruit is a fleshy sorosis of greenish-white to white or yellow color; up to 4 cm. in length in the material examined; mamellate on the surface, and consists of many coalescent drupes.

Fig. 1.—*Morinda citrifolia* L. leaf and flowering branch of plant growing in India. To right, below, a segment of root of same. $^1/_3$ ×

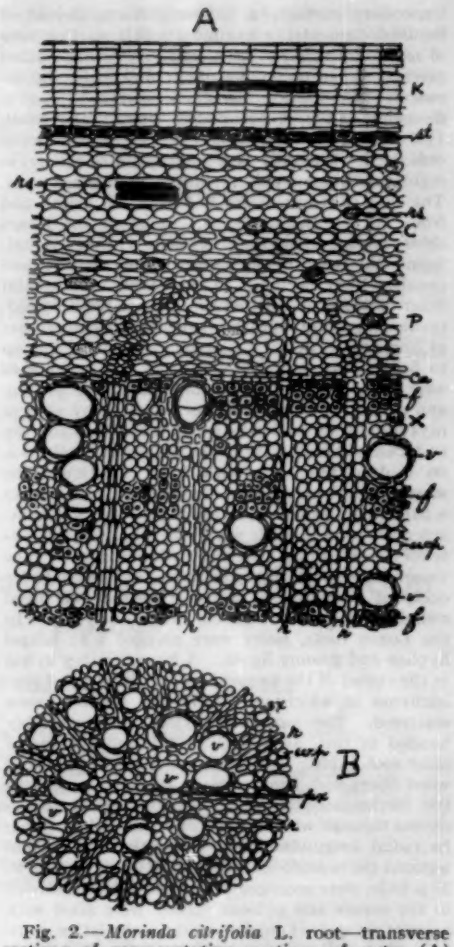

Fig. 2.—*Morinda citrifolia* L. root—transverse sections of representative portions of outer (A) and central (B) regions. *K*—cork with stone cells (*st*) occurring in outer, central and inner layers; *C*—phelloderm (secondary cortex); *rs*—raphide sacs; *P*—phloem; *Ca*—cambium; *X*—secondary xylem; *f*—groups of wood fibers; *v*—vessels; *r*—vascular rays; *wp*—wood parenchyma; *px*—primary xylem; *sx*—secondary xylem. (Magnified)

Physical Characteristics of Root.—The roots examined ranged from elongated conical roots with many thin rootlets toward the distal end to broader segments of thicker roots, the latter constituting most of the drug imported into this country. The commercial drug occurs in cylindrical to subcylindrical segments, some being curved, 2–15 cm. in length and up to 5 cm. in width, occasionally up to 9 cm. in width at the crown. Externally it is reddish brown, pale yellow where abraded, marked by irregular longitudinal wrinkles, some of the thicker pieces exhibiting transverse lenticels, circular root scars and transverse fissures. Internally it shows a narrow purplish brown bark, a broad yellow wood, and hard texture. Its odor is indistinct; its taste slightly sweet. Upon boiling the root in water and filtering, a yellow filtrate is found. On the addition of an alkaline solution to the filtrate, a reddish color develops, indicating anthraquinones.

Histology of *M. citrifolia* L. root.—Transverse sections of the distal portion of the rootlet, less than 0.8 mm. in diameter, exhibited primary dicotyl root structure with a triarch radial bundle. In transverse sections 2–2.6 mm. in diameter, the epidermis and the primary cortex were in the process of exfoliation, and a phellem of 2 layers of suberized walled cells had been formed by the phellogen. Beneath this occurred a very narrow phelloderm

(secondary cortex); a narrow phloem, devoid of lignified elements; a cambium and a narrow zone of secondary xylem; and a relatively broad, central primary xylem occupying about four-fifths the diameter of the rootlet. In a cross section 13 mm. in diameter, the bark occupied 2.5 mm.; the wood, 11.5 mm. The cork zone here was up to 18 layers of cells deep. The cells were rectangular to square, in regular radial arrangement, and with suberized walls. The cork cells were from 19μ to 66μ in length and from 11μ to 20μ in depth. Some of the layers showed ovate to oblong stone cells with nearly uniformly thickened lignified walls having distinct pore canals. They occurred in the outer, middle, and inner cork zones and frequently extended a considerable length through the layers, but isolated ones also occurred. All of the sections of thicker roots up to 5 cm. possessed this stone cork. Raphide sacs were numerous, scattered throughout the phloem and cortex. The phloem was traversed by phloem rays usually up to 3 cells wide, but occasionally up to 4, rarely 5, cells wide. The phloem ray cells took on a deep red color with KOH solution. The secondary xylem consisted of numerous wood wedges separated by xylem rays of straight to curved character whose cells walls were pitted and whose contents consisted of starch and anthraquinones. The vessels were mostly isolated in the wedges, but also occurred in groups of 2 to 4; usually arranged radially. A number of them exhibited tylosis. In the Luzon roots, many were plugged with fungal hyphae and gummy lignin. A large primary xylem in the center of the section showed pitted wood parenchyma in which vessels, mostly isolated, were scattered. The secondary xylem was invariably banded in this, as well as in broader sections of older root parts. The bands consisted of groups of wood fibers with lignified walls arranged in concentric circles between which occurred wood parenchyma through which vessels and tracheids coursed. In radial longitudinal and tangential longitudinal sections the raphide sacs, up to 120 μ long and up to 38 μ wide, were arranged singly and in vertical rows in the cortex and phloem. They were filled with acicular crystals of calcium oxalate, the latter up to 180 μ in length. In tangential sections, the sieve tubes showed laterally placed sieve plates. The xylem rays were mostly 1- to 3-cells wide, occasionally 1- to 4-cells wide. The vessels possessed closely set bordered pits and oblique ends with large circular openings in the perforation plates. They measured up to 418 μ in length and up to 152 μ, very rarely up to 200 μ, in width. The tracheids were mostly shorter, but like the vessels possessed closely set, bordered pits. Those measured were up to 240 μ in length and up to 102 μ in width. The wood parenchyma was abundant, its cells being up to 162 μ in length and 42 μ in width. It contained starch grains and anthraquinones. The wood fibers were straight to curved, some of them showed irregular lobes or teeth toward one or both ends. They were nonseptate. Their ends were mostly pointed and their walls lignified, showing oblique pits. These fibers were up to 1380 μ in length and up to 38 μ in width.

Sections of the roots from Luzon showed the presence of branched, septated hyphae of an endotrophic fungus which had penetrated into cells of all of the tissues, even occurring within the lumina of vessels, tracheids, and wood fibers. The fungus was probably mycorrhizal in origin.

Powdered *M. citrifolia* Root.—This was brown to reddish—or purplish brown—and possessed a slightly sweet taste. Under the microscope it showed: numerous fragments of wood fibers crossed by cells of xylem rays—the fibers with irregularly thickened, lignified walls and with narrow to broad lumina; numerous starch grains—single to 2- to 3-compound, the individual grains spheroidal, plano-convex, angular convex, pyriform and ovoid, the larger grains with a distinct centric hilum, the grains up to 26 μ in diameter and showing in the larger grains polarization crosses when examined under polarized light; scattered fragments of vessels and tracheids with closely set circular bordered pits; fragments of vascular rays with pitted walls; scattered fragments of cork whose cells are polygonal to rectangular with suberized walls; scattered cork stone cells with nearly uniformly thickened, lignified walls; numerous acicular crystals of calcium oxalate; and many fragments of elongate starch-bearing wood parenchyma. When mounted in 2% KOH solution, the tissue fragments took on a pink to red color, due to the solution of anthraquinones within the parenchyma cells and rays flowing out into the mount.

Work is in progress on a second paper in which the results of chemical and pharmacological studies on this root will be reported.

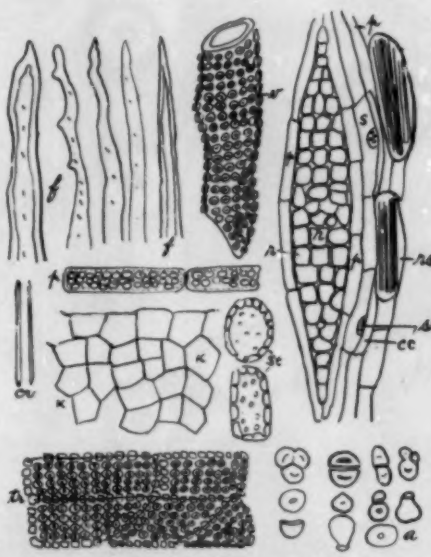

Fig. 3.—Histological elements occurring in longitudinal sections and in powdered *Morinda citrifolia* L. root. *f*—ends of wood fibers; *v*—vessel with closely set bordered pits; *r*—vascular ray of phloem region with adjacent tissues as seen in longitudinal tangential section; *p*—phloem parenchyma; *si*—sieve tube; *cc*—companion cell; *ra*—raphide sac with acicular crystals of calcium oxalate; *cr*—acicular crystals; *p*—starch parenchyma; *K*—cork; *St*—stone cells from cork region; *tr*—tracheids with bordered pits; *a*—starch grains (magnified).

SUMMARY

1. A brief history is given of the drug and its new clinical use in Viet Nam as a hypotensive and anticongestant.

2. A description is given of the plant materials and methods used in this study of the root of *Morinda citrifolia* which came from Thailand, India, and the Philippines.

3. A description is given of *Morinda citrifolia* L. based mostly on herbarium materials used by the author.

4. The physical characteristics of the root and the commercial drug are described.

5. The histology of the root of *M. citrifolia* is discussed. Among the microscopical features are the occurrence of stone cells in the cork, the absence of lignified elements in the cortex and phloem, a banded xylem composed of concentrically arranged groups of wood fibers, the sieve plates on the lateral walls of the sieve tubes, and vessels and tracheids with closely set circular bordered pits on their walls.

6. The occurrence of anthraquinones in the parenchyma and vascular rays as reported by Tunmann (4) is verified.

7. The powered root of *M. citrifolia* is described.

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Modification of Rates of Gastrointestinal Absorption of Drugs I. Amines*

By CHESTER J. CAVALLITO and ROSEMARY JEWELL

Tannates of several therapeutic amines have been prepared and shown to be reproducible compositions containing one part of amine for each galloylgallic acid moiety of tannic acid. Dialysis experiments were conducted to determine the influence of a number of factors on the rate of release of diffusible amine from the relatively insoluble complex. In the presence of aqueous solutions of electrolytes amines are slowly released from their tannates and the rate increases with the $[H^+]$. Polygalacturonic acid can reduce the rate of release of free amine from the tannate, particularly at lower pH. The application of these principles to the preparation of oral repository forms of therapeutic amines is discussed.

ALMOST ANY THERAPEUTICALLY active compound has some period of action during which there exists the optimum condition of desired response with least embarrassment of undesirable symptoms possible for that particular drug. If it were possible to provide a drug in such form that its rates of absorption and loss were suitably balanced to a more sustained level of optimum action, material improvement in therapy would result. The goal of much of the effort expended in the search for new drugs has been to find compounds which more effectively possess this characteristic. While success of such efforts at improvement is underscored by the rapid rate of obsolescence of products in the pharmaceutical industry, it is recognized that many of the older drugs for which no adequate replacement has been found and some of the newer ones would perform more satisfactorily if it were possible only to extend their period of optimum response. In the last several years a number of new approaches to this problem has been evident in the area of oral therapy. Although the oral route of administration is by far the most convenient, it is also the most difficult to control because of the variations in gastrointestinal environment among different individuals, with the same individual at different times and in different portions of the gastrointestinal tract.

Many medicinal agents are organic amines or their salts. If one desires a long response, the dosage of amine required is usually so high that for some period of time undesirable symptoms of over-dosage may result. Among parenteral preparations, the use of salts or complexes of low solubility has made possible more uniform and prolonged responses to medication. During the past few years, several oral preparations have

* Received June 29, 1957 from The Research Laboratories, Irwin, Neisler & Co., Decatur, Ill.

been introduced which utilize relatively insoluble forms of a drug under conditions which also provide more uniform and prolonged responses. In our laboratories the complexing agent which has been found to be particularly useful for amines is tannic acid. The favorable results which have been obtained clinically (1) with the use of amphetamine tannate[1] in providing oral repository amphetamine has led to the preparation and study of other amine tannates.

Tannates of many organic bases are of low aqueous solubility, but in the gastrointestinal tract they will release the base for absorption. Although the release is not contingent upon any specific pH, the rate is greater in environments of low pH than under neutral or slightly alkaline conditions. Some practical implications of this are discussed later.

Tannates of a variety of types of amine therapeutic agents have been made in these laboratories from tannic acid N.F. (2). A surprising characteristic of such preparations has been their relative uniformity of composition. Gallotannic acid may be idealized structurally as the pentadigallate ester of glucose, although the N.F. preparation is undoubtedly a mixture of closely related depside glucosides. In the tannate complexes the resulting products have a ratio of one amine for each digallyl moiety of the tannic acid.

Since the purpose of preparing oral repository forms of drugs is to provide, usually in the human, a longer period of optimum response, the evaluation of ultimate significance depends upon the performance of the preparation in practice. Recently there have been several reports describing *in vitro* procedures for measuring relative rates of release of amines from complexes with anionic polymers (3, 4). Such studies of the properties of these products may be of some value in gaining further understanding of their action and in providing approaches for improvements, but it is appreciated that release rate measurements obtained from arbitrary *in vitro* experiments can be only suggestive of behavior in humans.

EXPERIMENTAL

Preparation of Amine Tannates.—The preparative procedures vary slightly with the solubility of the amine, but a convenient method was found to be the following. The amine base was dissolved in a small volume of an alcohol (methyl, ethyl or propyl alcohols) and for each molar equivalent of base there was added 374 Gm. of tannic acid N.F. in a solution of the alcohol. This represents a 10% excess of one-fifth the molecular weight (1701) of pentadigallyl glucose, $C_{76}H_{52}O_{46}$. The mixture was diluted with an excess of ice water to complete precipitation of the tannate. The resulting precipitate was filtered off, washed thoroughly with ice water, and dried in a vacuum oven to yield an amorphous solid varying from nearly white to yellow in color.

The free amine base may be used or may be liberated by addition of the calculated quantity of sodium or potassium hydroxide to alcohol or aqueous-alcohol solution of the amine salt. Phenethylamines, such as amphetamine, form tannates which are quite soluble in methanol and which are prepared in concentrated solution prior to dilution with ice water. Alkaloids, such as atropine and morphine, on the other hand, form less soluble tannates in alcohol which precipitate when prepared in concentrated solutions, although incompletely without dilution with water. Antihistaminics of the dimethylaminoalkylpyridine type react quite cleanly, presumably with the more basic amino group to form monobasic complexes. With the less soluble tannates, such as those of morphine, codeine, atropine, etc., a nearly quantitative recovery of base is obtained.

TABLE I.—COMPOSITION OF TANNATES

Base	% Basic N in Compositions: Tannate, found	Calc'd. for Compositions Base:Tannic Acid Ratios 4:1	5:1	6:1
Amphetamine	2.8	2.50	2.95	3.35
Morphine	2.21	1.97	2.24	2.46
Atropine	2.16	1.96	2.22	2.45
Prophenpyridamine	4.74	4.21	4.82	5.34

The therapeutic base content of the tannate complex was determined by titration of the product in glacial acetic acid with standard perchloric acid solution. The basic nitrogen contents of representative types of amine tannates are shown in Table I. There also are shown the theoretical basic nitrogen contents of ratios of 4, 5 and 6 to 1, respectively, of base to tannic acid (assumed $C_{76}H_{52}O_{46}$). It is seen that the 5 to 1 ratio best represents the compositions.

The 5 to 1 ratio of amine to tannic acid appears to provide the least soluble composition. Preparations formed in which the amine ratios were smaller than this yielded more water-soluble products.

Release of Amines from Tannates.—The general procedure used for measuring release of amine from its insoluble tannate was as follows. A weighed amount of finely powdered amine tannate (or other salt for comparison) equivalent to 50 mg. of amine base was added to 100 ml. of the aqueous test medium in a Type SS Visking tubing[2] (presoaked in water for thirty minutes) which had been tied at one end to form an open sac 1⅞ inches in diameter, 6 inches long. This was immersed in 225 ml. of aqueous test medium contained in a 400-ml. beaker

[1] Synathan®, brand of tanphetamin, Irwin, Neisler & Co.

[2] Seamless, regenerated cellulose dialysis tubing; average pore radius 24 A°. Visking Corporation, Chicago, Ill.

so that the liquid levels of the beaker and the sac contents were the same. The contents were stirred vigorously and continuously. Exploratory experiments were first carried out both with the beaker contents undergoing continuous replacement with fresh aqueous test medium (rate of 50 to 100 ml. per minute) and with the original solution retained for establishment of an equilibrium. At regular intervals, a 5-ml. sample was withdrawn from the dialysate or sac contents for analysis to determine the extent of dialysis of water-solubilized amine. Prophenpyridamine maleate was used to determine the difference in diffusion by these two procedures. After 1, 2, 3, 4 and 6 hours' dialysis, the amounts of amine dialyzed with continuous replacement of dialysate were approximately 15% greater than in experiments with dialysate unchanged. Because of ease of manipulation, and since the data are primarily of comparative value, the procedure was selected in which the dialysate was not replaced.

The results obtained from the dialysis experiments are listed in Table II. The ease of quantitative analysis led to the use of the antihistaminic agent, prophenpyridamine, in exploring the influence of several variables on availability of drug from tannate complex under the *in vitro* conditions. The 5-ml. aliquot removed for assay was transferred to a small separatory funnel to which was added 1 ml. of 2% aqueous sodium hydroxide solution. This solution was extracted three times, each with 5 ml. of chloroform. The filtered, combined chloroform extracts were shaken out with 5 ml. of 0.05 *N* aqueous HCl. The absorbance (with suitable dilution, if necessary, to 1–5 mg. per 100 ml.) was measured at 260 mμ and the concentration determined from a standard curve prepared for prophenpyridamine.

A few comparative experiments were carried out with morphine in which the quantitative assay procedure was as follows. A 5-ml. aliquot was transferred to a small separatory funnel to which was added 2 ml. of 5% aqueous sodium bicarbonate solution. The procedure previously described for prophenpyridamine was then followed excepting that instead of chloroform, a 4 to 1 (by volume) chloroform-*n*-butyl alcohol mixture was used. The absorbance was read at 285 mμ within a preferred concentration range of 2–10 mg. per 100 ml.

Among the factors considered as possibly influencing release of an amine from its tannate complex were pH and electrolytes. Potassium phosphate buffers (0.01 molar) of pH 3 to 9 were used. The rate of diffusion of prophenpyridamine as its maleate salt was found to be essentially the same in distilled water as in the buffers. Prophenpyridamine tannate suspended in distilled water showed very little release of amine from solution and dialysis. The buffer electrolytes had an appreciable solubilizing effect which increased with lower pH values. Experiments were conducted at 25°; a few run at 38° showed only a slight increase in rate of amine release.

Tannic acid itself can diffuse through the Visking membrane. Its rate of diffusion, however, is appreciably slower than that of the dissolved amine. After solution in an electrolyte medium, the tannate molecule would not be expected to offer much hindrance to escape of the amine. In practice it has been found advantageous to include pectic or polygalacturonic[3] acids in oral compositions containing amine tannates. This polyanionic natural polymer provides an additional means for influencing the rate of release of free amine. (Mechanical as well as physical-chemical influences probably play a part.) The polygalacturonic acid was found to be

[3] Exchange Lemon Products Company, Corona, Calif.

TABLE II.—DIALYSIS OF AMINES

Compound	Medium	Retardant	% Dialyzed of Equilibrium Distribution[a] — Hours Dialyzed — 1	2	3	4	6	12	24
Prophenpyridamine Maleate	Water[b]	.....	24	33	45	55	68	..	..
Prophenpyridamine Tannate	Water	.....	..	<1	..	9	14	..	..
Prophenpyridamine Tannate	Buffer, pH 3	.....	19	28	37	49	64	83	95
Prophenpyridamine Tannate	Buffer, pH 5	.....	12	16	21	30	47	65	81
Prophenpyridamine Tannate	Buffer, pH 7	.....	8	10	14	18	23	35	51
Prophenpyridamine Tannate	Buffer, pH 9	.....	<1	7	9	13	19	30	49
Prophenpyridamine Tannate	Buffer, pH 3	Polygalacturonic Acid	12	19	22	31	41	51	73
Prophenpyridamine Tannate	Buffer pH 5	Polygalacturonic Acid	8	15	18	24	32	45	62
Prophenpyridamine Tannate	Buffer, pH 7	Polygalacturonic Acid	..	12	15	19	26	36	47
Prophenpyridamine Tannate	Buffer, pH 9	Polygalacturonic Acid	<1	9	13	18	23	31	40
Prophenpyridamine Maleate	Buffer, pH 3	Polygalacturonic Acid	20	29	37	46	56	..	..
Tannic Acid[c]	Buffers pH 3–7	.....	7	13	19	26	37	..	..
Morphine Sulfate	Buffers pH 3–9	.....	24	36	45	54	67	..	100
Morphine Tannate	Buffer, pH 3	.....	16	27	38	44	58	80	90
Morphine Tannate	Buffer, pH 7	.....	9	19	24	30	43	62	77

[a] Per cent in dialysate of that concentration of amine which would be present if all of that placed in the dialysis sac had become equally distributed throughout the total 325 ml. of fluid.
[b] Distilled water; 0.01 molar potassium phosphate buffers of pH 3 to 9 have no significant influence on these results.
[c] Tannic acid diffusion; no ami .

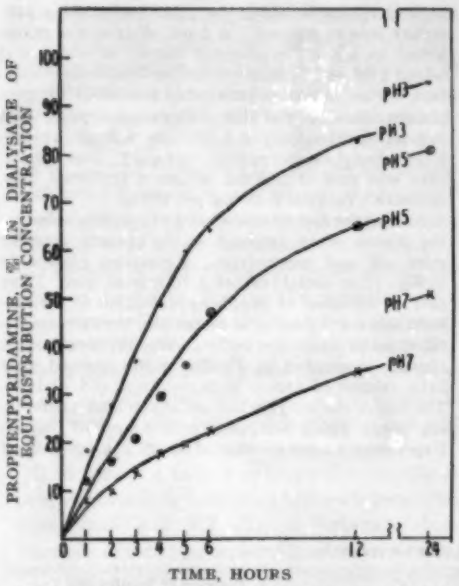

Fig. 1.—Prophenpyridamine Tannate. Influence of pH. Per cent dialysed of that concentration of prophenpyridamine which would be present in the dialysate if all of the prophenpyridamine tannate placed in the dialysis sac had become equally distributed.

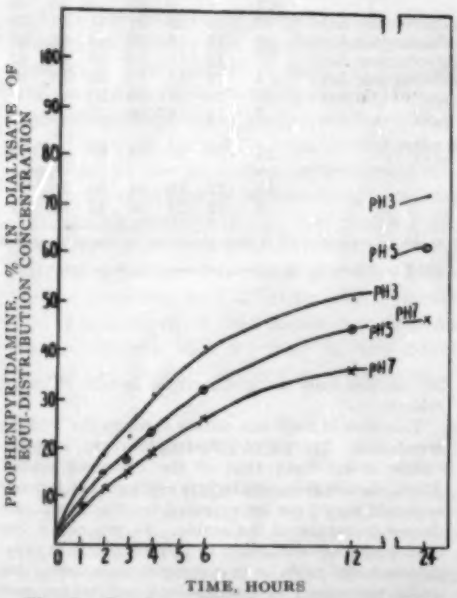

Fig. 2.—Prophenpyridamine Tannate + Polygalacturonic Acid. Influence of pH. Per cent dialysed of that concentration of prophenpyridamine which would be present in the dialysate if all of the prophenpyridamine tannate placed in the dialysis sac had become equally distributed.

nearly free of material diffusible through the membrane. Controls were run with the polygalacturonic acid and a correction made for its U.V. absorption. The influence of a 1% suspension on amine diffusion was measured after adjusting the suspension to several pH values with 0.1 *N* HCl or NaOH. A significant retardant action is evident at pH 3 and 5 with practically no effect at 7 and 9. The polygalacturonic acid is thus a more effective retardant of the amine at lower pH values; tannates are inherently less soluble in neutral or slightly alkaline environments.

A soluble morphine salt was shown to have about the same rate of diffusion as the soluble antihistaminic. The influence of pH on morphine tannate was analogous to that shown by the prophenpyridamine tannate.

DISCUSSION

It has been well known for many years that alkaloids form water-insoluble precipitates with tannins. There have been relatively few investigations of these compositions and these usually with rather crude preparations. The tannates derived from therapeutic amines and tannic acid N. F. have shown surprisingly uniform compositions. Recently, they have been applied in the preparation of oral repository forms of therapeutic amines. Although individual tannates vary considerably in solubility, amines of therapeutic interest generally are sufficiently large that their tannates are relatively insoluble in water. It has been shown that the presence of electrolytes and lowering of pH increases the rate of release of amine from tannate. Addition of polygalacturonic acid to the media can reduce appreciably the rate of dialyses of amine, particularly in environments of lower pH in which the tannates would usually be more soluble. An increase in $[H^+]$ would increase the concentration of carboxyl groups in the polycarboxylic retardant which would be available for salt formation and bonding with the amine. In practice, polygalacturonic acid is used concomitantly with the tannate to help provide the rate of initial release of amine found to be indicated by responses in humans. With the tannates, all components ultimately become individually dissolved and the therapeutic amine becomes totally available.

Sympathomimetic amines bound to insoluble cationic exchange resins (5, 6) also have been used as oral repository preparations. The mechanism of bonding of amines to insoluble cation exchange resins is apparently complex (7) and other variables are involved in addition to some of those influencing rate of release of amines from tannates.

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Modification of Rates of Gastrointestinal Absorption of Drugs II. Quaternary Ammonium Salts*

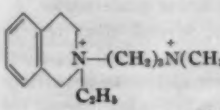
By CHESTER J. CAVALLITO and THOMAS B. O'DELL

From physical-chemical and physiological bases, several factors had been rationalized as possibly influencing oral absorption of quaternaries. These originally were evaluated in animals and later with *in vitro* studies. It has been found that in animals, lowering of intestinal pH, the administration of certain sterol acids or the addition of biologically inactive quaternaries all improve the oral responses to a quaternary hypotensive agent. *In vitro* studies have supported suggested explanations for some of these effects. Practical applications are currently under evaluation.

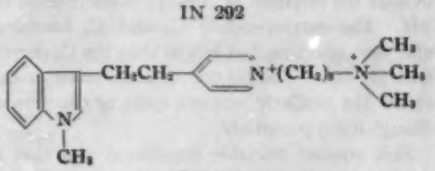
IN THE PRECEDING ARTICLE (1) there were discussed approaches to the control of rates of oral absorption of therapeutic amines in which the problem was to prevent too rapid initial absorption and to prolong the period of time during which drug action would be maintained. With quaternary ammonium salts, a significant and often limiting factor in oral therapy is their irregular and usually poor absorption from the gastrointestinal tract. Several techniques have been developed in our laboratories which, based on evidence from physical measurements and biological responses, result in improvement in oral effectiveness of quaternaries.

During the past several years, many types of diquaternary ammonium salts have been tested in animals in these laboratories for not only their biological activities by parenteral routes but also for their relative oral effectiveness. Ratios of oral to intravenous equipotent doses have been determined in animals and occasionally in the human. The variations have ranged from about 10 to 1 to several hundred times to 1 ratios between oral and intravenous effective doses. That these ratios are usually high has been well recognized. It is generally assumed that the ionized character of quaternaries renders many living membranes difficultly permeable to them. Presumably, anionic groups present in these membranes retain the cations by electrostatic bonding and displacement is slow by an ion exchange mechanism. Ruth Levine and coworkers (2), in an investigation of absorption and excretion rates of quaternaries in animals, have suggested a plausible hypothesis that mucins of the gastrointestinal tract act as barriers to quaternary absorption by formation of stable complexes. Irrespective of the exact location of the chemical structures barring quaternary absorption from the gut, there are several possible mechanisms for circumventing adsorption and facilitating absorption.

The quaternaries used in these studies were two unsymmetric bisquaternary hypotensive agents, IN 292 (3a,b) and IN 391 (4a,b), which showed about 20 to 1 ratios between oral and intravenous effective dosages in humans (5) and in dogs. Anything useful in increasing permeability of the gut to quaternaries must be physiologically innocuous. One of the classes of compounds considered was that of the bile acids.



IN 292



IN 391

These are known to influence oral absorption of a number of substances. It was rationalized that, in solution, a salt complex formed between a diquaternary and a bile acid might be less readily subject to ion exchange and fixation of quaternary at anionic receptors in the intestinal wall. Experiments in animals demonstrated a significant favorable influence of cholic acid, for example, on oral effectiveness of IN 292. This was confirmed in humans (5) in which 150 to 200 mg. of cholic acid doubled the oral effectiveness of similar quantities of IN 292. The limitation in practice was that the bile acids would produce a laxative effect which was not always desirable.

The next approach was rationalized as follows. The relative affinities of quaternaries for adsorption at sites of loss in the intestine probably are unrelated to the activities of those quaternaries

* Received June 29, 1957 from The Research Laboratories, Irwin, Neisler & Co., Decatur, Ill.

The authors are indebted to Mrs. Martha D. Napoli and Mrs. Rosemary Jewell for technical assistance and to Dr. A. P. Gray for some of the chemicals.

as hypotensive agents. It might, therefore, be possible to administer, together with the hypotensive agent, a quaternary which was physiologically inert but which could effectively compete at sites of loss in the gut, thereby resulting in greater availability for absorption of free unadsorbed therapeutic quaternary. Since the hypotensive agents were compounds of type

$$Q>\overset{+}{N}—CH_2CH_2CH_2\overset{+}{N}(CH_3)_3$$

in which Q is a large group, an ideal potentiator might be a salt of the simple compound

$$(CH_3)_3\overset{+}{N}—CH_2CH_2CH_2\overset{+}{N}(CH_3)_3$$

IN 414

which biologically is relatively inert. Experiments in animals and humans showed that this diquaternary was appreciably effective in improving responses to oral medication with IN 292 or IN 391. Concomitant intravenous administration in animals of IN 414 with the hypotensive agents showed no significant potentiation by this route. The mechanism appears to involve potentiation by competition in the gastrointestinal tract at sites of loss of the active quaternaries.

In addition to IN 414, several other closely related, biologically relatively inert, diquaternaries were tested for their ability to potentiate in dogs the response to "orally" administered IN 391. The corresponding C_2 and C_4 homologs also were effective, but less so than the C_3 derivative, IN 414. The bis-triethylammonium analog of IN 414 simularly was not quite as effective although it did potentiate.

Still another variable considered was that of pH and its influence on relative bonding affinities of quaternaries for polyanionic receptors. It was found that with increasing hydrogen ion concentrations, appreciably less fixation of quaternary at polyanionic receptors was evident. Oral effectiveness of IN 391 in dogs was found to be significantly increased by increasing the acidity of contents of loops of intestine.

In the experimental section there are discussed data which support the preceding conclusions and permit speculations as to implications of these studies.

EXPERIMENTAL

In vitro Diffusion Measurements.—The general procedure employed in measuring rates of diffusion of quaternary ammonium compound was essentially the same as that described in the preceding article (1). The quaternary used was IN 391 which showed sharp U.V. absorption maxima at λ255 mμ and 288 mμ. Optical density-concentration standard curves for the compound permitted accurate determinations of quantities of the order of 2 to 5 mg. per 100 ml. At regular intervals, aliquots were taken of the dialysate and the IN 391 cation content was determined directly or after suitable dilution with water. The chloride salt of the quaternary was used throughout; several of the experiments which were repeated with bromide salt gave the same results. These salts in distilled water gave solutions of pH 6.0.

The data obtained are summarized in Table I. All experiments were conducted at least in duplicate. To 100 ml. of the solvent medium in the dialysis sac there was added 60 mg. of IN 391 chloride (50 mg. of IN 391 cation). The same solvent medium (225 ml.) less drug was added to the beaker on the outside of the sac (dialysate). At the time intervals indicated, 5-ml. aliquots were removed from the dialysate for analysis. Among the variables considered as possibly influencing diffusion of quaternaries through the inert, semipermeable membrane were pH and presence of electrolytes. The quaternary was equally distributed within and outside of the dialysis sac in about six hours with water as the solvent medium. The use of 0.01 *M* NaCl solution significantly reduced the rate of diffusion of the quaternary as did also 0.01 *M* potassium phosphate buffer. The greater retarding influence of the phosphate buffer than of sodium chloride is probably related to the greater concentration of ions in this buffer solution. Variations in pH between 4 and 8 had no influence on diffusion rate.

TABLE I.—IN 391 DIFFUSION

Solvent Medium	Retardant	Potentiator	pH	% Dialyzed of Equi-distribution Concn.[a] Hours Dialyzed 1	2	3	4	5	6
Water	.....	.....	6	41	58	..	88	95	98
0.01 *M* NaCl	.....	.....	6	29	..	56	68	79	82
0.01 *M* K Phosphate buffer	.....	.....	4–8	23	37	45	57	64	70
Water	Mucin	.....	4	17	..	42	52	55	61
Water	Mucin	.....	6	12	21	27	34	39	41
Water	Mucin	.....	8	7	12	22	27	31	36
Water	Mucin	100 mg. Citric Acid	3.5	11	27	..	46	57	68
Water	Mucin	100 mg. Tartaric Acid	3.3	14	26	..	55	64	74
Water	Mucin	IN 414 (60 mg.)	6	20	31	42	47	53	58
Water	Mucin	IN 414 (90 mg.)	6	29	42	..	58	64	66
Water	Pectic Acid	.....	3	<1	6	8	10	11	11
Water	Pectic Acid	IN 414 (90 mg.)	3	9	18	24	30	34	37

[a] Per cent in dialysate of that concentration of IN 391 which would be present if all of that placed in the dialysis sac had become equally distributed throughout the total 325 ml. of fluid.

One of the structures which has been implicated as possibly retarding cation absorption is the mucin lining of the gastrointestinal tract (2). Experiments were carried out with a 1% suspension of Gastric Mucin Powder[1] in water as the solvent medium for IN 391 in the dialysis sac. This preparation in distilled water had a pH of 6.0. The influence of pH was observed by adjusting to pH 4 with dilute HCl or to 8 with dilute NaOH. Mucin was shown to have a marked influence in retarding dialysis of the quaternary and the influence was particularly greater at higher pH values. The dialysis membrane was relatively impermeable to the mucin; however, a small amount of material slowly diffused through which showed some absorption in the U.V. analytical range. The diffusible impurities were dialyzed out of a 1% suspension of the mucin for a twenty-four hour period preceding use of the suspension in these experiments. The addition of 100 mg. of either citric or tartaric acid to the mucin medium also showed the influence of added organic acids (lower pH) on mucin binding of the quaternary.

In the preceding article, the influence of polygalacturonic (purified pectic) acid was evaluated as a retardant for amine absorption. This polycarboxylic acid appears to have a very marked bonding capacity for the quaternary.

In Figure 1, there are shown the relative influences of electrolytes, mucin and pectic acid on rates of IN 391 diffusion; in Fig. 2, the influence of pH on mucin binding is demonstrated.

The ability of a biologically relatively inert quaternary (IN 414) to increase oral effectiveness of IN 391 had been predicated on the assumption that the sites of loss of the therapeutic quaternary in the gastrointestinal tract would be blocked by adsorption of the biologically inactive potentiator. The influence of addition of 60 mg. or 90 mg. of IN 414 chloride to the IN 391-mucin or IN 391-pectic acid combinations is evident from the table or Fig. 3. The IN 414 significantly reduces the apparent binding of the IN 391 to the anionic polymers.

Pharmacological Activity Measurements.—The effectiveness of additives in potentiating the "oral" activity of quaternaries was determined as follows. Dogs were anesthetized with pentobarbital sodium and the blood pressure measured directly from the common carotid artery. The quaternary salts, in aqueous solution, were administered directly into the upper third of the small intestine. The procedure was then repeated (different dogs) using the quaternary in combination with the additive agent. Most experiments were repeated at least once. Insoluble acid additives were dissolved in 1.0 to 2.0 ml. of 2% aqueous sodium bicarbonate solution. Responses were evaluated on the basis of per cent fall in blood pressure from normal and duration of fall before return of blood pressure to pre-drug level or to level of maximum recovery. Results are summarized in Table II.

The influence of pH on intestinal absorption was measured by the same procedure in dogs. IN 391 was administered in 10 ml. of 0.1 molar potassium phosphate buffers of pH 4.0 and 8.4 by injection into the small intestine at a point just below the distal end of the pancreas. Tying off a six-inch loop of intestine at the area of injection enhanced the re-

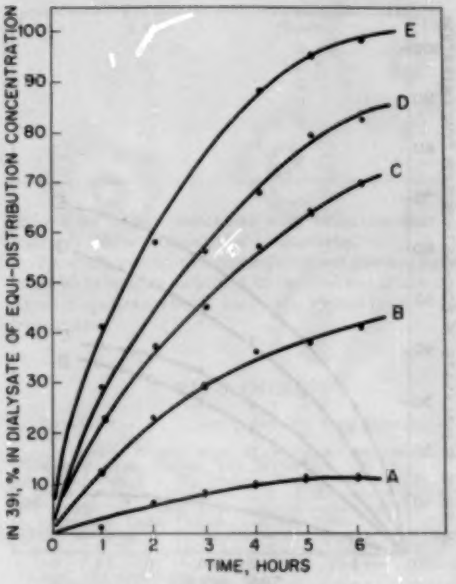


Fig. 1—Dialysis of IN 391 from: (*A*) 1% pectic acid suspension in water, (*B*) 1% mucin suspension in water, (*C*) 0.01 *M* phosphate buffer, (*D*) 0.01 *M* sodium chloride solution, (*E*) water (distilled) (Table I).

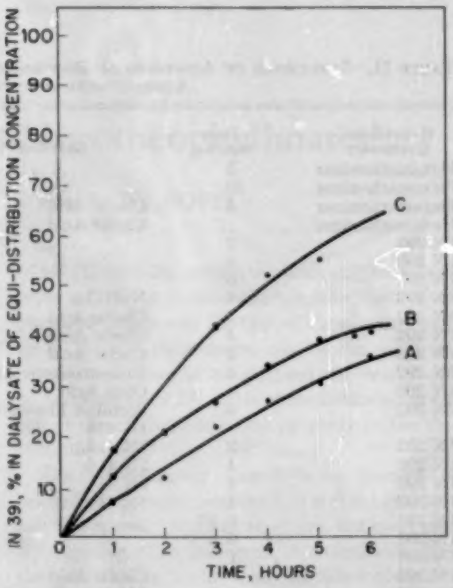


Fig. 2—Dialysis of IN 391 from 1% mucin suspension in water at pH (*A*) 8, (*B*) 6, (*C*) 4 (Table I).

[1] Gastric Mucin (N.N.R., 1957, p. 386).

sponses to some extent as might be expected. Results are outlined in Table III.

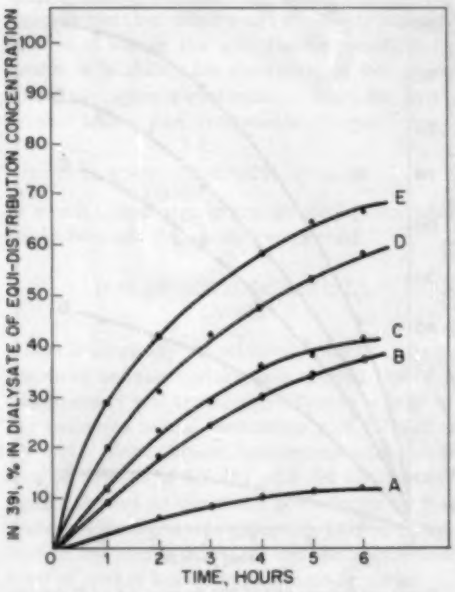


Fig. 3—Dialysis of IN 391 from: (*A*) 1% pectic acid suspension in water, (*B*) as in (*A*) but with IN 414 added, (*C*) 1% mucin suspension in water, (*D*) as in (*C*) but with 60 mg. IN 414, (*E*) as in (*C*) but with 90 mg. IN 414 (Table I).

DISCUSSION

The erratic and often poor absorption of quaternaries by the oral route may be related to a number of gastrointestinal environmental variables. The mucin lining could well be a major barrier to their absorption from the gastrointestinal tract. The barrier effect would be expected to increase and absorption of quaternary become poorer as the environment became less acid. This might result in a more rapid absorption of quaternaries in the upper part of the gastrointestinal tract with poorer absorption later. The influence of pH on mucin binding might explain why the major part of the oral absorption of a poorly absorbed quaternary appears to occur fairly quickly (2). As the intestinal contents become less acid, quaternary binding by polyanionic food components (such as pectic acids) as well as the mucins could become more significant. The improved oral absorption of quaternaries by fasting individuals (6) might result from not only less loss of quaternary by binding to food components and dilution but possibly also from less neutralization of secreted acid. One might speculate that quaternaries which reduce gastric acid secretion may indirectly curtail absorption of later doses and lead to apparent tolerance. Reserpine has been reported to increase gastric acid secretion (7) although this is uncertain (8). Drugs which increase gastrointestinal acidity might produce some potentiation of quaternaries by improving their absorption.

The magnitude of the influence of other ions on binding of quaternaries to mucins or to other parts of the gastrointestinal wall is difficult to assess. Living membranes usually are selectively permeable to ions and different electrolytes might vary considerably in their influence on absorption of quaternaries.

TABLE II.—INFLUENCE OF ADDITIVES ON RESPONSES TO QUATERNARY AMMONIUM HYPOTENSIVE AGENTS ADMINISTERED INTO THE INTESTINE OF DOGS

Hypotensive Quaternary	Dose mg./Kg.	Additive	Dose mg./Kg.	Blood Press. % Fall	Duration, Hours[a]
Hexamethonium	5	.....	..	45	1
Hexamethonium	10	.....	..	50	2.5
Hexamethonium	5	Cholic Acid	10	50	4.5
Hexamethonium	..	Cholic Acid	10	0	0
IN 292	2	.....	..	15	1
IN 292	4	.....	..	20	2.5
IN 292	6	.....	..	25	3
IN 292	4	$NaHCO_3$	2	30	3
IN 292	2	Cholic Acid	10	35	>3.5
IN 292	4	Cholic Acid	5	35	>5
IN 292	4	Cholic Acid	10	60	>4
IN 292	4	Dehydrocholic Acid	10	50–70	>4
IN 292	4	Oleic Acid	10	20	3
IN 292	4	Sorbitan Monostearate	10	25	2.5
..	..	IN 414	2	0	0
IN 292	2	IN 414	1	65	4.5
IN 292	4	IN 414	0.5	60	>2
IN 292	4	IN 414	1	65	>4
IN 292	4	IN 414	2	65	>3
IN 391	0.35	.....	..	20	3
IN 391	0.5	.....	..	30	>4
IN 391	1	.....	..	50	>4
IN 391	0.5	Cholic Acid	10	55	>3
IN 391	0.5	IN 414	0.5	50	>3.5

[a] Durations greater than three to four hours cannot be reliably measured in anesthetized dogs.

TABLE III.—INFLUENCE OF pH ON RESPONSES TO HYPOTENSIVE QUATERNARY IN 391 ADMINISTERED INTO THE INTESTINE OF DOGS

Intestine open or tied loop	Buffer pH	mg./Kg. IN 391	Blood Press., % Fall	Duration, Hours
Open	4.0	0.35	20	3
Open	4.0	0.5	30	>2.5
Open	8.4	0.35	0	0
Open	8.4	0.5	0	0
Loop	4.0	0.5	45	4
Loop	8.4	0.5	0	0
Loop	8.4	1.0	35	4

The mutual influences of ions on their intestinal absorption is apparently complex (9).

Experiments also have demonstrated that two steroid acids significantly increase the responses (particularly duration) to the two quaternary hypotensive agents. The surface-active, lipophilic oleic acid and sorbitan monostearate have practically no influence on the responses to the quaternaries. Although cholic acid lowers blood pressure when injected parenterally, it and the related conjugated bile acids do not appear to enter the circulation when administered orally and have no influence on blood pressure (10, 11). Control experiments in the present series show no effects on blood pressure from "oral" cholic acid alone. The mechanism of this influence on quaternaries is not clearly understood. The steroid acids might increase permeability of the intestinal wall; they might have a mild mucolytic action thereby reducing the barrier effects of intestinal mucins; they may form salts with quaternaries and provide enough anion molecular bulk to reduce the frequency of ion exchange. The last mechanism is not unreasonable and might permit more facile passage of the quaternary salt of the steroid acid across a lipophilic living membrane. In any event, the implication is that individual variations in biliary secretions may influence quantitatively the oral absorption of quaternaries.

To what extent the gastrointestinal environment may be favorably modified to facilitate oral absorption of quaternaries is currently under further investigation.

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Preparation of Some 8-Nitrotheophyllinates*

By W. MOROZOWICH and F. W. BOPE†

The 8-nitrotheophyllinates of aniline, aminopyrine, diphenhydramine, atropine, and cocaine were prepared. The presence of two melting points with the aniline salt indicates its unstability. Attempts to prepare the 8-nitrotheophyllinate of antipyrine were unsuccessful.

NUMEROUS SALTS of 8-halotheophyllines with basic compounds, particularly the narcotic bases (1) and the antihistaminics (2), have been reported. The 8-chlorotheophylline salt of diphenhydramine was the first of these compounds to be made. Drowsiness, one of the main untoward reactions of antihistaminics, was found to be diminished (3) in these compounds by virtue of the CNS stimulating property of the theophylline moiety.

The 8-substituted theophyllines possess an acidic hydrogen on position 7, which is capable of salt formation. Since nitro groups are more electro-negative than halogens, 8-nitrotheophylline should be more acidic than the 8-halotheophyllines.

* Received May 13, 1957, from the College of Pharmacy, Ohio State University, Columbus, Ohio.
Based on a thesis presented by W. Morozowich to the Graduate School, Ohio State University, in partial fulfillment of the requirements for the degree Master of Science.
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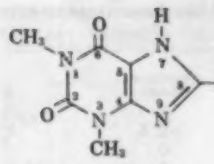

8-Nitrotheophylline

Increased acidity should result in more stable salts with less chance of precipitation of the free xanthine derivative in the stomach.

Dusel, *et al.* (4), have prepared the choline salts of theophylline, 8-chlorotheophylline, and 8-bromotheophylline. The oral LD_{50}'s of these compounds were found to range between 500–850 mg./Kg. The LD_{50} of the choline salt of 8-nitrotheophylline was nonlethal at 5,000 mg./-Kg.

The purpose of this work was to prepare the 8-nitrotheophyllinates of some antihistaminic and analgesic compounds and to attempt synthesis of the aniline and the aminopyrine salts which could not be prepared as the 8-chlorotheophyllinates (1).

EXPERIMENTAL

8-Nitrotheophylline was prepared by nitrating theophylline in glacial acetic acid according to the method of Dusel, *et al.* (4). Potentiometric titration of 8-nitrotheophylline with standard sodium hydroxide solution established the pKa at 3.58. The m. p. of 8-nitrotheophylline was 282–283° with decomposition.

Aminopyrine 8-Nitrotheophyllinate.—A sample of 0.450 Gm. (0.0200 mole) of 8-nitrotheophylline was refluxed with 0.502 Gm. (0.0216 mole) of aminopyrine in 150 ml. of 99% isopropyl alcohol. After refluxing for one hour the clear yellow solution was concentrated to 30 ml. by distillation, then cooled to −5°. The yellow crystals obtained were recrystallized twice from 99% isopropyl alcohol, then dried over P_2O_5 at 90° and 3 mm. for three hours. The m. p. was 194–196°. Microscopically the crystals were clustered together in the form of yellow rosettes. Titration of a 0.0405 Gm. sample required 1.001 equivalents of standard NaOH, indicating that the salt consists of a 1:1 ratio of 8-nitrotheophylline to aminopyrine. Infrared spectrographs were obtained for the starting materials and characteristic absorptions were identified in the final compound.

The following compounds were similarly prepared: aniline-, diphenhydramine-, cocaine-, and atropine 8-nitrotheophyllinate. Crystal structure, C, H analysis and melting points of the compounds are reported in Table I. All of the compounds were found to be 1:1 ratios of base to 8-nitrotheophylline.

DISCUSSION

The m. p. of the aniline 8-nitrotheophyllinate taken conventionally on a Fisher-Johns apparatus was 279–283°, approximately the same as that of 8-nitrotheophylline. However, when the compound was dropped onto a preheated melting point block it melted when the temperature was above 155°, then solidified, and melted again at 279–283°. This indicates that the compound is quite unstable and probably reverts to the starting materials when above 155°.

TABLE I.—8-NITROTHEOPHYLLINATES

Compound	M.p.[a]	Crystal Nature	Formula	C, H (Calcd.)	C, H (Found)[b]	Yield, %
Aminopyrine 8-Nitrotheophyllinate	194–196	Yellow rosettes	$C_{20}H_{24}N_8O_5$	52.63, 5.30	52.57, 5.40	45
Diphenhydramine 8-Nitrotheophyllinate	197–198	Yellow rods	$C_{24}H_{28}N_6H_5$	59.99, 5.87	60.20, 5.82	63
Cocaine 8-Nitrotheophyllinate	103–111	Yellow rods	$C_{24}H_{28}N_6O_9$	54.54, 5.34	54.42, 5.54	48
Atropine 8-Nitrotheophyllinate	202–206	Yellow rosettes	$C_{21}H_{29}N_6O_7$	56.02, 5.87	56.10, 5.85	43
Aniline[c] 8-Nitrotheophyllinate	155[d]	Yellow parallelograms and some elongated hexagonals	$C_{13}H_{14}N_6O_4$	49.06, 4.43	50.86, 5.74	45

[a] Melting points were taken on a Fisher-Johns melting point block.
[b] Carbon-hydrogen analyses were carried out by Schwartzkopf Laboratories, New York, N. Y.
[c] Composition in doubt due to discrepancies in C, H analyses and to crystal nature. See Discussion.
[d] When dropped onto a hot melting point block, it melts above 155°, then solidifies, and melts again at 279–283°, which is approximately the same as for 8-nitrotheophylline.

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A Study of the Effect of Certain Pharmaceutical Materials on Color Stability*

By ROY KURAMOTO, LEON LACHMAN, and JACK COOPER

The influence of several pharmaceutical materials on the fading of the certified color, FD&C Blue No. 2, has been investigated. It has been found that sugars such as dextrose, lactose, and sucrose increase the rate of fading, whereas sugar alcohols such as mannitol and sorbitol do not appreciably affect the rate. However, if trace amounts of strong reducing catalysts remain in the sugar alcohol after being crystallized, they significantly affect color stability. In general, antioxidants such as hydroquinone, *p*-hydroxypropiophenone and hydroquinone monomethyl ether were found not to appreciably retard the fading due to reducing sugars. In the case of NDGA, however, the rate of fading due to dextrose is substantially reduced. It appears that the rate of fading of the above color is catalyzed in the presence of reducing compounds.

ALTHOUGH COLOR FADING of pharmaceutical preparations appears to be a common occurrence, no previous reports on the mechanism of the reaction seems to have been published in the literature. It has been a common practice in the pharmaceutical industry to discard the use of a specific color if it is found to be unstable in a given formulation. This was done without determining the agent or agents which were responsible for this reaction. This approach is now becoming more difficult to utilize since the number of certified dyes has been diminishing due to recent FDA investigations and subsequent rulings. Therefore, it appeared to be desirable to investigate the actual mechanism of the reaction in order to possibly retard the rate of fading.

The certified dye, FD&C Blue No. 2, an indigo sulfonate, was utilized in this investigation. This dye was chosen since it was found to be very unstable in the presence of many pharmaceutical ingredients or when subjected to different environmental conditions. It was decided to study the rate of fading of this dye in solution rather than in formulations such as tablets, suspensions, etc.; since this form is more amenable to stability investigation and chemical analysis. In addition, the instability of this dye would lend itself to a thorough study within a reasonable length of time.

From the chemical structure of the dye and actual experience with color stability in product formulations, it appeared that the fading was due to an oxidation-reduction reaction as well as to a dependency on light intensity. However, in this study no attempt was made to isolate the contribution of the light intensity factor to the overall reaction.

In order to substantiate the above reasoning, the effect on color stability caused by various reducing and nonreducing sugars, sugar alcohols, antioxidants, and other compounds was evaluated. Experimental data indicate that sugars such as dextrose, lactose, and sucrose increase the rate of fading; whereas sugar alcohols such as sorbitol and mannitol do not appreciably affect the rate. However, if trace amounts of strong reducing catalysts remain in the sugar alcohol after being crystallized, it significantly affects color stability. In general, antioxidants such as hydroquinone, *p*-hydroxypropiophenone and hydroquinone monomethyl ether were found not to appreciably retard the fading due to reducing sugars. In the case of NDGA, however, the rate of fading due to dextrose is substantially reduced.

PREVIOUS STUDIES

A search of the literature reveals that although there are numerous reports on the fading of vat dyes in the textile industry, there is a conspicuous lack of reports on the mechanism of color fading in pharmaceutical formulations. One of the few investigations on color stability in pharmaceutical preparations has been reported on by Garrett and Carper (1). They recently reported on the color stability of a liquid multi-sulfa preparation by kinetic investigation of the thermally accelerated degradation of the color. They did not attempt to determine the mechanism of the reaction or the ingredients which were responsible for the fading. Their main purpose was to predict the shelf color stability of this formulation in a brief period of three and one-half weeks, which they appeared to have accomplished successfully.

The effect of light intensity on textile color stability has been reported on by Taylor and Pracejus (2) and others (3–8). In general, most investigators report that color fading in textiles is dependent on light intensity. Desai and Vaidya (5) indicated that dyes such as Brilliant Green, Malachite Green, Crystal Violet, Rosaniline, and others were stabilized against sunlight to varying degrees by the addition

*Received May 3, 1957, from the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.
Presented to the Scientific Section, A. Ph. A., New York meeting, April–May 1957.
The authors are sincerely grateful to Nancy Colangelo and Robert Wolf for their assistance in this study.

of copper sulfate. Results of Taylor and Pracejus (2) indicate that the fading of colored textiles by daylight is principally due to radiant energy in the visible spectrum.

The food industry, in many respects, has similar problems with color stability as do the pharmaceutical companies. For example, an interesting study by Pruthi and Lal (9) on the color of citrus juices has shown that sweetening agents present in the product tended to accelerate the color changes. This is in agreement with the observations found with pharmaceutical formulations containing similar type sweetening agents.

There have been numerous publications on the oxidation-reduction reactions of various dyes. It appears that the color fading of indigo is due to the formation of a leuco compound preceded by an unstable intermediate (10–12). In addition, it has been shown that metallic ions catalyze the oxidation or reduction of indigo type colors (13–15).

It is apparent from the above cited references that dyes are readily affected by reducing and oxidizing agents as well as by light intensity. Since many pharmaceutical formulations contain colorants as well as various ingredients which possess reducing or oxidizing properties, it appeared desirable to investigate the color stability of these preparations. This approach appeared essential in order to determine the mechanism of the reaction from which one can postulate a possible method of retarding the rate of fading.

EXPERIMENTAL

Materials Used.—Sucrose U. S. P., dextrose U. S. P., lactose U. S. P., mannitol N. F., sorbitol (Atlas Powder Co.), sorbitol solution N. F. (Sorbo®, Atlas Powder Co.), hydroquinone (Merck & Co.), nordihydroguaiaretic acid (NDGA, K & K Laboratories, Long Island City, N. Y.), hydroquinone monomethyl ether (Eastman Chemical Products, Inc.), *p*-hydroxypropiophenone (Eastman Kodak Co.), FD&C Blue No. 2 (Calco Chemical Division, Lot 68652), and phosphate buffer solution, pH 6.6.

Equipment.—Beckman spectrophotometer model DU, Beckman recording spectrophotometer model DK-1, and Beckman pH meter model G.

Procedure.—Aqueous buffer solutions (pH 6.6) containing 0.15% dye, 0.15% dye + 5% sugar or sugar alcohol, 0.15% dye + 5% dextrose + 0.1% chemical additives, and 0.15% dye + 0.1% chemical additives were prepared and investigated for their color stability. These samples were stored at room temperature and exposed to ordinary room illumination. At designated time intervals, aliquot samples were removed and assayed spectrophotometrically for residual color at 610 mμ where $E_{1\%,\,1cm} = 248$.

RESULTS AND DISCUSSION

The stability of FD&C Blue No. 2 in a buffered aqueous solution was investigated in the presence of several materials commonly found in pharmaceutical preparations. These studies were buffered at pH 6.6 in order to maintain a constant hydrogen ion concentration as well as to approximate the pH of a 0.15% aqueous solution of the dye.

The influence of various sugars on the rate of fading of the dye is presented in Table I and Figs. 1 and 2. It can readily be seen from Fig. 1 that dextrose substantially increases the rate of fading after a period of induction. This "lag time" is probably due to the formation of a semiquinone intermediate which is very unstable. A straight line plot after the initial lag time indicates an apparent first order reaction with respect to the dye concentration.

TABLE I.—INFLUENCE OF SUGARS ON THE RATE OF FADING OF FD&C BLUE NO. 2 AT PH 6.6 AND AT ROOM TEMPERATURE

Sugar	Rate × 10^3 ($Days^{-1}$)
Dextrose	19.60
Sucrose	9.20
Lactose	9.78

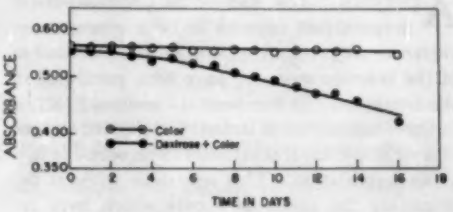


Fig. 1.—A plot showing the influence of dextrose on the rate of fading of FD&C Blue No. 2 where the upper line represents the rate of fading of the dye in the absence of dextrose.

From Fig. 2 it is apparent that lactose and sucrose affect the rate of fading in a manner similar to dextrose. However, it appears that the monosaccharide has a greater influence on the color instability than the disaccharides. Although sucrose is a nonreducing sugar, it still causes fading, but to a lesser degree than lactose. This is essentially due to the hydrolysis of the sucrose to form dextrose and fructose both of which have reducing groups.

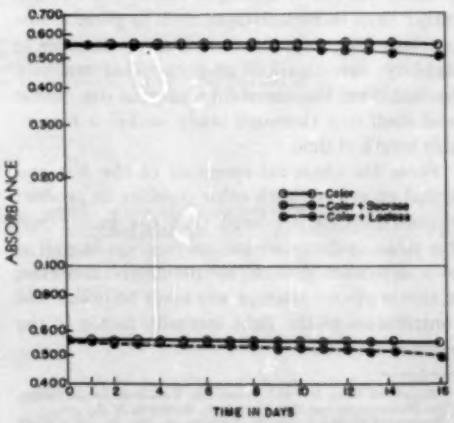


Fig. 2.—A plot comparing the effect of sucrose and lactose on the rate of color fading.

From the data presented in Table II and Fig. 3, it seems that sorbitol and mannitol do not appreciably affect the rate. However, it can be seen from the curve of sorbitol Lot 634 that some batches of sorbitol significantly affect the rate of color disappearance. This may be due to the presence of trace amounts of strong reducing catalysts which were not completely removed during the purification stage. Although the actual method of preparation and purification of this particular batch of sorbitol is not readily available, two methods of preparation have been reported in the literature (16, 17) which utilize strong reducing catalysts such as nickel and iron. The curve representing sorbitol Lot 634 does not show an induction period and this can be attributed to the more rapid formation of the semiquinone which is subsequently converted to the leuco form. This strongly indicates that such materials should be adequately tested by the Quality Control Division before being used in pharmaceutical formulations containing colorants.

TABLE II. INFLUENCE OF SUGAR ALCOHOLS ON THE RATE OF FADING OF FD&C BLUE NO. 2 AT PH 6.6 AND AT ROOM TEMPERATURE

Sugar Alcohols	Rate × 10^3 (Days^{-1})
Sorbitol (Lot 1709)	2.04
Sorbitol Solution N. F.	7.36
Sorbitol (Lot 634)	27.60
Mannitol	7.36

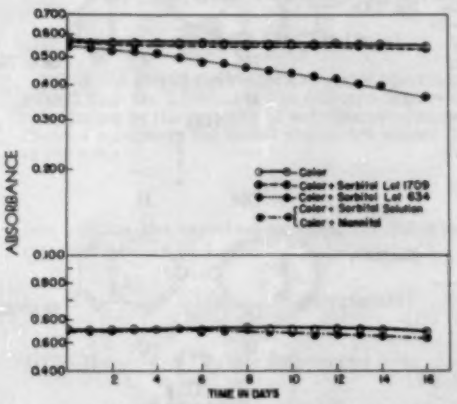


Fig. 3.—The influence of various sugar alcohols on the rate of fading of FD&C Blue No. 2.

In order to determine the effect of some antioxidants and related chemicals on the rate of color fading, various solutions containing these agents were investigated. The information obtained from these studies is presented in Table III and Figs. 4–7. It is apparent from data in Table III and Fig. 4 that the rate of color fading in the presence of dextrose and hydroquinone monomethyl ether is approximately the sum of the rates of each compound. However, in the case of hydroquinone, as shown in Fig. 5 and Table III, the rate of fading appears to be greater than the additive effect of

TABLE III.—INFLUENCE OF ANTIOXIDANTS AND RELATED CHEMICALS ON THE RATE OF FADING OF FD&C BLUE NO. 2 AT PH 6.6 AND AT ROOM TEMPERATURE

Additive	Rate × 10^3 (Days^{-1})
Hydroquinone monomethyl ether	6.90
Hydroquinone monomethyl ether + dextrose	28.50
Hydroquinone	7.36
Hydroquinone + dextrose	33.40
p-Hydroxypropiophenone	0.00
p-Hydroxypropiophenone + dextrose	19.60
NDGA	9.20
NDGA + dextrose	9.20

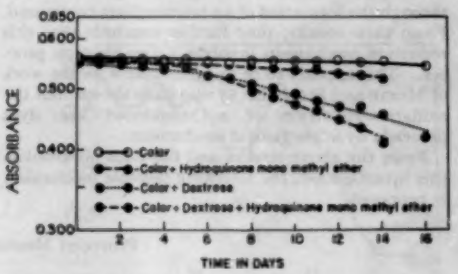


Fig. 4.—The effect of hydroquinone monomethyl ether on the rate of color fading due to dextrose.

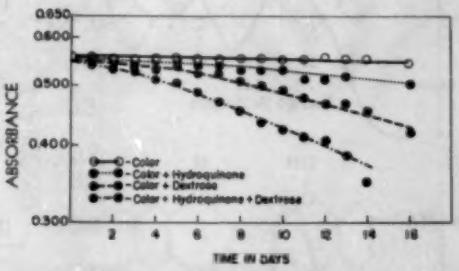


Fig. 5.—A graph indicating the influence of hydroquinone and dextrose on the rate of color fading.

dextrose plus hydroquinone. The rate is essentially equal to the sum of the rate due to dextrose plus twice the rate due to hydroquinone. This may be attributed to the two easily oxidizable groups present in the hydroquinone molecule. Whereas, with *p*-hydroxypropiophenone (Fig. 6), it can readily be seen from Table III that this compound did not contribute to the overall rate. This may be explained by the presence of a relatively strong electron attracting group on the molecule in the *para* position. In contrast to the above additives, the presence of NDGA in the color solutions retards the effect due to dextrose. It can be seen from Fig. 7 that the rate of fading is essentially due to the effect of NDGA and the dextrose does not appear to contribute to the overall effect. This may be explained

by the relative ease of oxidation of NDGA as compared to that of dextrose.

It can be seen from Figs. 8 and 9 that there is a significant change in the absorption spectrum both in the visible and UV regions. Figure 8 illustrates the changes in intensity of the color due to the extent of reduction to the colorless leuco form. However, in Fig. 9, the curves indicate a change in the structure of the dye molecule upon reduction. The absorption maximum at 285 mμ appears to decrease as the carbonyl group is reduced. This is further substantiated by the formation of a new maximum at 235 mμ which is probably due to the reduced form of the carbonyl group.

Mechanism.—A search of the literature did not appear to reveal a comprehensive mechanism for the reduction of indigo dyes. However, Appleton and Geake (12) indicate that indigo undergoes reduction through the formation of an intermediate compound. From their results, they further conclude that this reduction mechanism involves a two-electron process. This appears to be in agreement with the work of Moran and Stonehill (8) who have shown that the oxidation-reduction of anthraquinoid vat dyes proceeds by a free radical mechanism.

From the above studies and the data obtained in this investigation, the following possible mechanism is proposed:

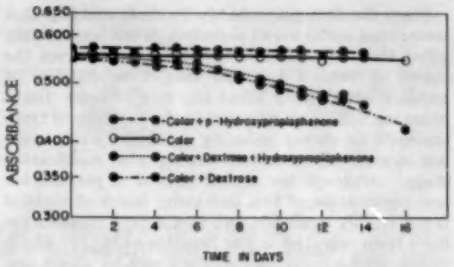


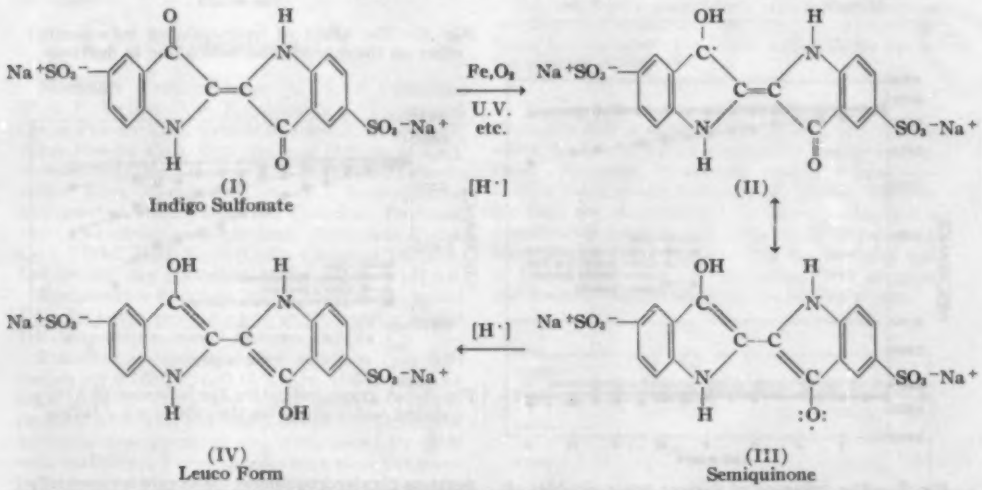
Fig. 6.—A plot showing the insignificant effect of *p*-hydroxypropiophenone on the rate of fading due to dextrose.

The semiquinone (II & III) gives only two of the several possible contributing forms to the resonance hybrid. However, it would appear that III is probably the most stable electronic configuration of this group.

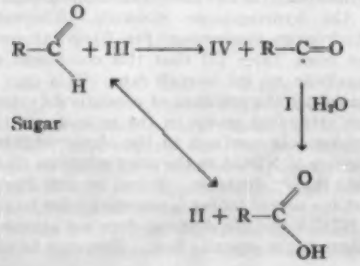
The induction period observed in this study is probably due to the build-up of the semiquinone which is subsequently reduced to the leuco form.

Derivation of the Rate Equation.—Assuming the above mechanism is representative of the reaction

Proposed Mechanism of Reaction



Propagation of Reaction



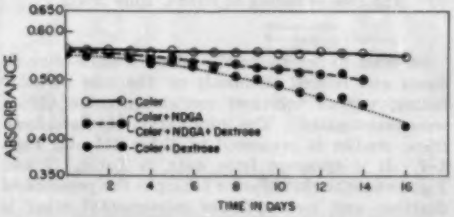


Fig. 7.—The retarding effect of nordihydroguaiaretic acid on the rate of fading of FD&C Blue No. 2 due to dextrose.

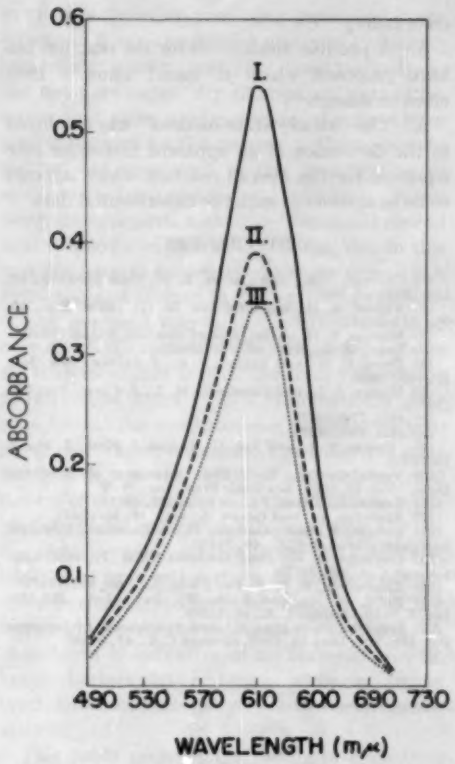


Fig. 8.—A plot of the visible absorption spectra of FD&C Blue No. 2 indicating the different degrees of color fading in the presence of reducing substances. Curve I represents the initial absorption maximum of the color.

and utilizing the experimental data, the following rate equation can be derived:

$$I + H\cdot \xrightarrow{k_1} III \text{ (unstable intermediate)} \quad \text{(Eq. 1)}$$

$$III + H\cdot \xrightarrow{k_2} IV \text{ (rate determining step)} \quad \text{(Eq. 2)}$$

$$\frac{d(IV)}{dt} = k_2 (III)(H\cdot) \quad \text{(Eq. 3)}$$

$$\frac{d(III)}{dt} = k_1 (I)(H\cdot) - k_2 (III)(H\cdot) \quad \text{(Eq. 4)}$$

Since III is assumed to be an unstable intermediate, the "steady state method" may be employed to compare the postulated mechanism with the empirical data. This method is applicable only to those cases in which the intermediates are unstable and, therefore, their concentrations are at all times much smaller than those of the reactants. For such cases, it is reasonable to postulate that after a time, which is very short relative to the half-time of the reaction, a steady state is attained and during the steady state the rate of change of the concentrations of the intermediates is negligibly small. On the basis of this assumption, one may set the time derivatives of the concentrations of each of the intermediates equal to zero.

If

$$\frac{d(III)}{dt} = 0, k_1 (I)(H\cdot) = k_2 (III)(H\cdot) \quad \text{(Eq. 5)}$$

Therefore:

$$\frac{d(IV)}{dt} = k_1 (I)(H\cdot) = \frac{-d(I)}{dt} \quad \text{(Eq. 6)}$$

Equation 6 is representative of the overall reaction which is given by the mechanism above.

Since the concentration of the reducing agent is so much greater than the dye concentration, it can

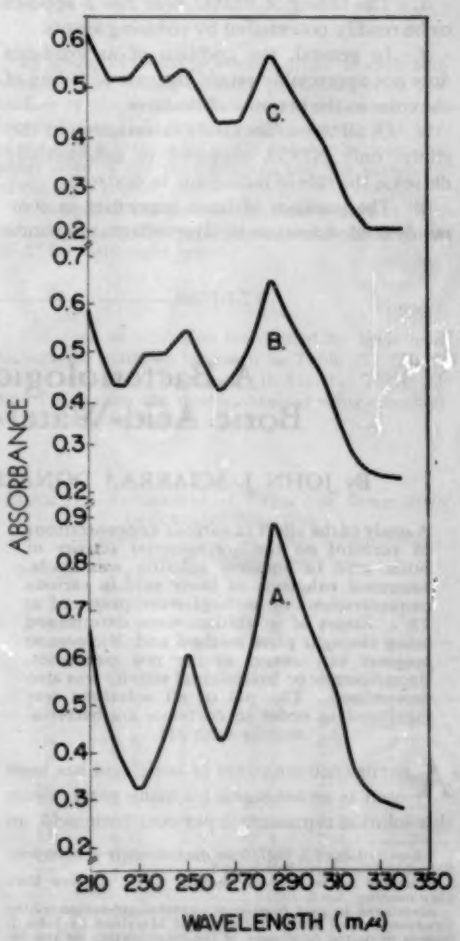


Fig. 9.—A plot of the ultraviolet absorption spectra of FD&C Blue No. 2 indicating a change in the spectra due to the extent of formation of the colorless leuco form. Curve A represents the initial absorption spectrum of the dye.

be assumed that the hydrogen free radical term in Eq. 6 is constant.

Then:

$$\frac{-d(\mathrm{I})}{dt} = k_3(\mathrm{I}) \qquad \text{(Eq. 7)}$$

This shows that the assumed mechanism is consistent with the observed first order kinetics.

SUMMARY AND CONCLUSIONS

The effect of several pharmaceutical materials on the rate of fading of FD&C Blue No. 2 has been investigated. From the experimental data obtained and the postulated mechanism, the following conclusions can be drawn:

1. The fading of FD&C Blue No. 2 appears to be readily potentiated by reducing agents.

2. In general, the addition of antioxidants does not appreciably retard the rate of fading of the color in the presence of dextrose.

3. Of all the antioxidants investigated in this study, only NDGA appeared to substantially decrease the rate of fading due to dextrose.

4. The presence of trace impurities in commonly used pharmaceutical ingredients may cause color fading.

5. A possible mechanism for the reaction has been proposed which is based upon a two-electron change.

6. The "steady state method" was employed in the derivation of an apparent first-order rate equation for the overall reaction which appears to be in agreement with the experimental data.

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A Bacteriological Evaluation of Boric Acid-Water-Sorbitol Solutions*

By JOHN J. SCIARRA,† DONALD E. SHAY,‡ and JOHN AUTIAN§

A study of the effect of various concentrations of sorbitol on the antibacterial activity of boric acid in aqueous solution was made. Saturated solutions of boric acid in various concentrations of sorbitol were prepared at 25°. Zones of inhibition were determined using the agar plate method and *Micrococcus pyogenes* var. *aureus* as the test organism. Bacteriostatic or bactericidal activity was also determined. The pH of all solutions was measured in order to correlate antibacterial activity with pH.

A SATURATED SOLUTION of boric acid has been used as an antiseptic for many years. Since this solution represents 5 per cent boric acid, an increase in concentration becomes impossible. It was previously reported that sorbitol in combination with boric acid will increase the solubility of this acid from 5 per cent to approximately 20 per cent by weight (1). It was hoped that this increased concentration of boric acid would prove to be a better antiseptic solution.

The antiseptic activity of boric acid in sorbitol solutions is possibly due to three factors: the increased concentration of boric acid, the antiseptic properties of the complex compound, and the decrease in pH of the solution.

Zipf and Stephan (2) reported that products of boric acid with aliphatic polyhydroxy compounds have a lower pH than boric acid and possess higher toxic effects on organisms.

Many methods have been used to test the antibacterial power of preparations designed for application to the body surfaces (3, 4). One of these, the phenol coefficient technique, is limited

* Received April 5, 1957, from the University of Maryland, School of Pharmacy, Baltimore 1.
Presented to the Scientific Section, A. PH. A., New York City meeting, April, 1957.
Abstracted in part from a dissertation submitted to the Graduate School of the University of Maryland by John J. Sciarra in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1957.
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to those substances possessing germicidal properties and is not an effective means of evaluating bacteriostatic substances (4). Such methods as: the wet filter-paper, dry filter-paper, agar-plate, serum agar-plate, agar cup-plate, etc., have been used in the past for this purpose. The agar-plate method has been used in this investigation to evaluate the boric acid-sorbitol solutions. When using the agar-plate technique, the cooled melted agar is inoculated with the test organism, in this case *Micrococcus pyogenes* var. *aureus*, poured in a Petri dish and allowed to harden. The test substance is placed into penicillinders which have been set on the surface of the agar, and the plates are then incubated at 37.5° for from twenty-four to forty-eight hours. Upon examination a clear zone around the test substance indicates no visible growth of the organism; the wider the zone the greater are the diffusion and antibacterial powers of the substance. If the substance is not inhibitory the organisms will grow over the entire plate, even in contact with the test substance. Samples of agar within the zone are then subcultured in a nutrient broth, incubated at 37.5° for forty-eight hours, and examined for turbidity. A clear broth is indicative of no bacterial growth, hence bactericidal activity; while a turbid broth indicates bacterial growth or bacteriostatic activity.

This paper presents the results of comparing the zones of inhibition manifested by boric acid alone and in combination with sorbitol.

EXPERIMENTAL

The media used in this experiment were sterile nutrient agar, pH 6.8 (B. B. L.); and sterile nutrient broth, pH 6.9 (B. B. L.).

The organism used was *Micrococcus pyogenes* var. *aureus* (F. D. A. strain 209). It was selected as the test organism since this is one of the bacteria used by the Federal Food and Drug Administration to test antibacterial substances. A 4-mm. loopful of organisms from a stock culture was transferred to a sterile tube of nutrient broth and incubated at 37.5° for twenty-four hours.

Solutions of sorbitol in distilled water were prepared containing from 0 to 70% by weight of sorbitol in increments of 5%. These solutions constituted the controls.

Solutions of boric acid in distilled water were prepared containing from 0 to 5% by weight of boric acid in increments of 0.5%.

The boric acid-sorbitol solutions were prepared according to the concentrations given in Table I.

The Petri dishes were prepared by liquefying the sterile medium, adding 1 ml. of a twenty-four-hour broth culture of the test organism when the medium cooled to 56° and then pouring into the Petri dishes in 20-ml. amounts. When the plates solidified four penicillinders were placed equidistant on the surface of the agar.

TABLE I.—CONCENTRATION OF BORIC ACID-SORBITOL SOLUTIONS

Solution No.	Boric Acid, %(w/w)	Sorbitol, % (w/w)	Water, % (w/w)
1	5.57	0.00	94.43
2	6.31	4.68	89.01
3	7.09	9.29	83.62
4	7.87	13.82	78.31
5	8.72	18.26	73.02
6	9.47	22.63	67.90
7	10.42	26.87	62.71
8	11.18	31.09	57.73
9	11.63	35.47	52.90
10	12.92	39.19	47.89
11	13.80	43.10	43.10
12	14.98	46.76	38.26
13	16.17	50.30	33.53
14	17.31	53.75	28.94
15	18.66	56.96	24.38

By means of sterile pipets five drops of each test solution were placed into the penicillinders on the surface of the Petri dishes. The dishes were then incubated at 37.5° for forty-eight hours. The size of the zone was recorded as the distance between the outer edge of the penicillinders and the periphery of the clear zone.

A sample of the agar in the clear zone was then subcultured in nutrient broth and incubated at 37.5° for forty-eight hours.

RESULTS

The zone of inhibition manifested by boric acid in sorbitol solutions is shown in Table II. These results are shown graphically in Fig. 1. Table III and Fig. 2 show the results obtained using solutions of boric acid in distilled water.

TABLE II.—ANTIBACTERIAL EFFECT OF BORIC ACID IN SORBITOL SOLUTION

Solution No.	Zone of Inhibition, mm. Detn. No. 1*	Detn. No. 2*	Detn. No. 3*	Av.	pH
1	4.3	8.1	8.8	7.1	3.80
2	4.4	9.0	8.4	7.3	2.90
3	6.9	7.6	8.3	7.6	2.50
4	5.4	8.3	5.4	6.4	2.35
5	5.6	8.0	4.9	6.2	2.20
6	6.5	8.0	5.9	6.8	2.15
7	6.6	8.1	5.3	6.7	2.10
8	5.6	7.4	5.1	6.0	2.00
9	7.1	7.9	6.1	7.1	1.90
10	8.2	7.4	7.1	7.6	1.85
11	6.3	8.3	6.9	7.2	1.75
12	6.3	8.3	7.1	7.2	1.70
13	6.0	8.9	7.4	7.4	1.70
14	6.0	9.4	7.3	7.3	1.60
15	7.1	9.5	8.1	8.1	1.60

* Average of eight determinations.

The control solutions of sorbitol alone did not inhibit the growth of *Micrococcus pyogenes* var. *aureus*. This observation is not surprising since

TABLE III.—ANTIBACTERIAL EFFECT OF BORIC ACID IN WATER

Boric Acid, Concn. % w/w	Av. Zone of Inhibition,[a] mm.	pH
0.0	0.0	7.20
0.5	2.0	6.35
1.0	3.4	5.85
1.5	4.6	5.50
2.0	5.9	5.10
2.5	6.4	4.85
3.0	6.8	4.55
3.5	7.9	4.45
4.0	8.6	4.30
4.5	8.9	4.15
5.0	9.7	3.95

[a] Average of eight determinations.

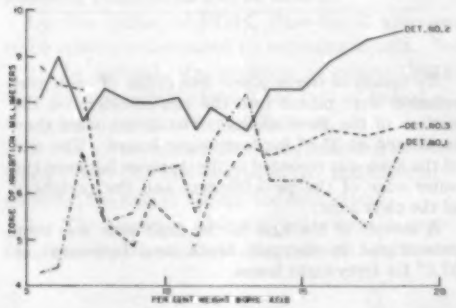

Fig. 1—Antibacterial activity of boric acid-sorbitol solution.

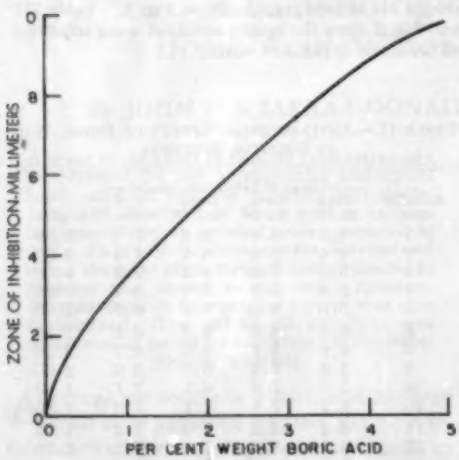

Fig. 2—Antibacterial activity of boric acid in water.

sorbitol is not antibacterial, in fact, it is sometimes used as a component of various media used for the growth of bacteria.

The results of the subculture of the clear zone of inhibition in nutrient broth showed boric acid in these concentrations, and when tested in this manner, exerted a bacteriostatic effect and not a bactericidal one. All of the tubes indicated bacterial growth after a forty-eight hour incubation period.

DISCUSSION OF RESULTS

The results suggest that the maximum antibacterial activity of boric acid occurs when used in a concentration of approximately 5%. Any further increase in concentration of boric acid, made possible by the addition of sorbitol, was not indicative of greater antibacterial activity. Apparently the sorbitol interferes in some manner with the antimicrobial activity of boric acid. No direct relationship was found to exist between the concentration of boric acid in sorbitol solution and the antibacterial effect. There were several solutions where the effectiveness decreased with an increase in concentration of boric acid. This decrease may be due to the formation of a complex compound between boric acid and sorbitol. The antibacterial activity of this new compound has been found to be less than that of a 5% boric acid solution in water.

In an attempt to understand the cause for the variation in activity of boric acid in the presence of sorbitol, the pH of the solution was measured. The pH was found to decrease with an increase in concentration of boric acid. This lowering of pH verified the presence of a more acidic compound but did not explain the various peaks shown in Fig. 1. Had there been an increase in pH for those solutions showing lesser antibacterial activity, the difference could be explained. It is known that a decrease in hydrogen-ion concentration for acidic substances will decrease antibacterial activity and an increase in hydrogen ion concentration will increase antibacterial activity (5).

A possible explanation, at this time, for these differences in antibacterial activity of boric acid solutions in the presence of sorbitol, may be that sorbitol, being a nutrient for bacteria, hinders the activity of boric acid. Further studies are indicated to explain the mechanism of this activity.

SUMMARY AND CONCLUSIONS

1. A study was made of the antibacterial activity of various solutions of boric acid in the presence of sorbitol.

2. Maximum antibacterial activity of boric acid occurred at about a 5 per cent concentration.

3. No relationship was found to exist between the concentration of boric acid in sorbitol solution and the antibacterial activity, nor between the pH and antibacterial activity. Further work is necessary to fully establish these observations.

4. Boric acid in sorbitol solution was found to be bacteriostatic rather than bactericidal.

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The Toxicity and Metabolism of Dihydroquercetin*

By ALBERT N. BOOTH and FLOYD DeEDS

Dihydroquercetin, which is closely related to quercetin in chemical structure, has been shown to be nontoxic when fed to albino rats at a dietary level of 1 per cent for long periods of time. Chromatographic examination of the urine of two human volunteers before and after the ingestion of 2 grams of dihydroquercetin demonstrated the conversion of the compound to 3,4-dihydroxyphenylacetic, *m*-hydroxyphenylacetic, and 3-methoxy-4-hydroxyphenylacetic acids. These same metabolites are excreted following oral administration of quercetin, or DOPA, to rats, rabbits, or humans.

THE ANTIOXIDANT PROPERTIES, physiological effects, and metabolic fate of rutin and its aglycone, quercetin, have been studied in this laboratory (1–8). The close structural relation of dihydroquercetin to quercetin (Fig. 1) invites comparison of the two compounds in regard to toxicity, antioxidant properties, physiological effects, and metabolism.

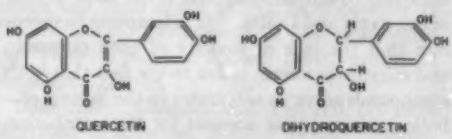

Fig. 1.—Structural formulas of quercetin and dihydroquercetin.

Quercetin occurs as the glycoside rutin in the buckwheat plant (*Fagopyreum esculentum* and *tartaricum*), in the leaves of *Eucalyptus macrorhyncha*, in the flower buds of the Chinese scholar tree (*Sophora japonica*), and as the rhamnoside quercitrin in the bark of the black oak tree (*Quercus velvutina*). Commercial quercetin has been prepared by acid hydrolysis of the material isolated from these sources (9–12). At present the most prolific source of dihydroquercetin is Douglas fir bark which contains 80 to 152 pounds per ton of bark (13). The ease of conversion of dihydroquercetin to quercetin (14) makes Douglas fir bark another potentially important source of the latter.

In a study of comparative abilities of a number of flavonoids to protect epinephrine against oxidation *in vitro* Wilson and DeEds (3) reported that 3 molar equivalents of dihydroquercetin provided about 0.8 the antioxidant effect afforded by 1 molar equivalent of quercetin. Because of the ease with which dihydroquercetin is oxidized to quercetin it was recently suspected that the antioxidant activity *in vitro* ascribed to dihydroquercetin may have been due to quercetin present as an impurity. Wilson has now shown that dihydroquercetin purified by repeated crystallization has negligible antioxidant protective action toward epinephrine *in vitro* (15). This does not necessarily militate against the possibility that dihydroquercetin may be converted to quercetin in the animal body and therefore produce the same physiological effects as quercetin. The observations on the metabolism of dihydroquercetin reported herein are in harmony with this suggestion. Moreover, Ambrose, Robbins, and DeEds (6) reported that dihydroquercetin, in common with rutin, quercitrin, and quercetin, provided some protection against severe acute frostbite in rabbits. Unpublished data obtained on 5 rabbits, using the technic described by Ambrose and DeEds (2, 4) demonstrated that dihydroquercetin decreased the responsiveness of cutaneous capillaries to acute local irritation.

EXPERIMENTAL

Chronic Toxicity.—For comparison with toxicity data reported on quercetin (7), the following long-term feeding experiment with dihydroquercetin was conducted. Twenty weanling albino rats of each sex from our stock colony served as controls on a basal diet having the following percentage composition: degerminated yellow corn meal, 73; crude casein, 10; linseed oil cake meal, 10; ground alfalfa, 2; bone ash, 1.5; sodium chloride, 0.5; and cod liver oil, U. S. P. 3. Ten weanling rats of each sex of the same strain were placed on dietary levels of 0.125, 0.25, 0.5, and 1.0% of dihydroquercetin incorporated in the same basal diet. Groups of five rats, all of one sex, were kept in cages with pine shavings as litter, and with free access to water and food at all times. The amount of dihydroquercetin available dictated the following feeding periods. At the end of 226 days, half of the animals receiving 1.0 and 0.5% of the compound and an equal number of controls were sacrificed; the remainder on these concentrations being sacrificed after 450 days. After 249 days, half of the animals on diets containing 0.25 and 0.125% and an equal number of controls were sacrificed, the remainder being continued for 650 days. The range of dosage levels employed was deemed adequate in view of the close chemical relationship of dihydroquercetin to quercetin and

* Received May 28, 1957, from Pharmacology Section, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif.

the reported lack of toxicity of the latter (7) at a dietary level of 1%.

At weekly intervals throughout the course of the experiment the rats were weighed individually and carefully examined. At this time the food consumption of each group of five rats was determined.

Throughout the course of the feeding test no abnormalities in appearance or behavior of the experimental animals as compared with the controls were observed. A rather high incidence of respiratory infection, as evidenced by rales, developed in the control animals as well as in those on the various dietary levels of dihydroquercetin. There were 12 deaths in a total of 120 rats during the experiment, but the deaths bore no relation to the treatment. As compared with the controls, the various dosage levels of dihydroquercetin had no effect on either food intake or rate of growth. The lack of any effect on growth is shown in Table I, which summarizes the average initial and final weights of rats on the control and experimental diets at the end of 226 days. No differences were found in body length or weights of liver, kidney, spleen, heart, adrenal, and testis between animals receiving 1% dihydroquercetin for 226 days and their appropriate controls.

TABLE I.—BODY WEIGHT GAIN OF RATS ON CONTROL AND DIHYDROQUERCETIN-CONTAINING DIETS FOR 226 DAYS

% Dihydroquercetin	Males		Females	
	Initial Av. Wt., Gm.	Final Av. Wt., Gm.	Initial Av. Wt., Gm.	Final Av. Wt. Gm.
Control	40	362	42	254
0.125	39	352	37	254
0.25	39	350	37	258
0.5	42	356	46	246
1.0	42	361	46	248

At 450 days five control rats of each sex, five males and four females receiving 1%, and four males and five females receiving 0.5% dihydroquercetin were sacrificed and examined for gross abnormalities. Paraffin sections of kidney, liver, heart, lung, testis, ovary, thyroid, hypophysis, adrenal, gut, pancreas, spleen, and bladder were stained with hematoxylin-eosin for histopathological examination. Lung consolidation was observed grossly in one control rat of each sex, in one rat of each sex receiving 1%, and in one female receiving 0.5% dihydroquercetin. Cysts were found on the liver and kidney of one control female, and on the uterus and ovary of another control. Bladder stones were found in the male controls and a kidney tumor in one male control.

Histopathological examination of the stained tissue sections showed that a majority of the control rats had mild focal pyelonephritis. The kidney tumor noted grossly in a male control was a tubular adenoma of questionable malignancy. The lung consolidations noted grossly were suppurative, and the cysts were of inflammatory origin. No changes of significance were found in any of the organs of male rats fed 0.5 and 1.0% dihydroquercetin, nor were any significant changes found in other organs of female rats fed 0.5% dihydroquercetin. The livers of the female rats fed 1.0% of the compound showed vacuoles, probably due to fat deposition, scattered throughout. With the possible exception of this vacuolization, neither the gross nor microscopic examination revealed any significant changes attributable to the dihydroquercetin.

Metabolic Fate.—Booth, Murray, Jones, and DeEds (8) have demonstrated that oral administration of quercetin to rats, rabbits, guinea pigs, and humans results in the urinary excretion of 3,4-dihydroxyphenylacetic acid, *m*-hydroxyphenylacetic acid, and 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid). It has also been shown by DeEds, Booth, and Jones (16) that these same metabolites are excreted following oral administration of DOPA (3,4-dihydroxyphenylalanine).

When the urine of two human volunteers was examined by the chromatographic procedures described previously (8), before and after the ingestion of 2 Gm. of dihydroquercetin, it was found that the same metabolites were excreted as in similar experiments with quercetin and with DOPA.

SUMMARY

No significant toxic results were observed from dihydroquercetin,[1] which is closely related to quercetin in chemical structure, when fed to albino rats at a dietary level of 1% for long periods of time. It has also been shown that the metabolic fate of dihydroquercetin is the same as that of quercetin and DOPA. It is tempting to speculate that the lack of toxicity of both quercetin and dihydroquercetin is due to the fact that both compounds serve as substrates in the same metabolic pathways that account for the metabolism of DOPA.

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[1] It is a pleasure to thank the Oregon Forest Products Laboratory for a liberal supply of dihydroquercetin which made this investigation possible.

Catalase in Cunninghamella Blakesleeana H-334*

By ARLINGTON A. FORIST

Techniques for the determination of crystalline catalase activity have been extended to crude cell homogenates and employed in an examination of catalase in *Cunninghamella blakesleeana* H-334. Variation in catalase content during growth of this microorganism indicates that the older, mature cells are the catalase producers. Catalase does not appear to be involved in the 11β-hydroxylation of steroids by *Cunninghamella blakesleeana* H-334.

CUNNINGHAMELLA BLAKESLEEANA strain H-334 (ATCC 9245) contains enzyme systems capable of directly introducing the 11β-hydroxyl group, necessary for biological activity, into the steroid nucleus. Incubation of Reichstein's compound S (11-desoxy-17α-hydroxycorticosterone) with this microorganism yields hydrocortisone (17α-hydroxy-corticosterone) (1). An examination of enzymes present in this microorganism has included a study of its catalase. The present report describes the method of determination and some of the properties of catalase in *Cunninghamella blakesleeana* H-334 along with the change in catalase content during growth of the microorganism.

Bonnichsen, Chance, and Theorell (2) have described an excellent method for the measurement of crystalline catalase activity based on a high enzyme-low substrate ratio and a short reaction time. By this procedure, disappearance of hydrogen peroxide was observed to follow a first order rate. The present communication describes an extension of this technique to the measurement of catalatic activity in crude systems.

EXPERIMENTAL

Cultures.—Most of the *C. blakesleeana* H-334 samples examined were grown in shake flasks on a complex medium (soybean meal, 5 Gm.; brewer's yeast, Pabst, 5 Gm.; dextrose, 20 Gm.; NaCl, 5 Gm.; KH_2PO_4, 5 Gm.; tap water, 1,000 cc.; pH adjusted to 6.4) as described by Mann, *et al.* (3). Some samples were also grown on Czapek-Dox solution ($NaNO_3$, 3.0 Gm.; $K_2HPO_4 \cdot 3H_2O$, 1.3 Gm.; $MgSO_4$, 0.5 Gm.; KCl, 0.5 Gm.; $FeSO_4 \cdot 7H_2O$, 0.01 Gm.; sucrose, 30 Gm.; distilled water, 1,000 cc.; pH adjusted to 7.2) (4).

Preparation of Crude Catalase.—The mycelia of *C. blakesleeana* H-334, usually pellet-like, were separated from the medium by filtration on cheesecloth or by centrifugation, washed with distilled water on the filter or by centrifugation, and resuspended in cold *M*/15 phosphate buffer, pH 7.0. The cell suspension was then homogenized with a pestle homogenizer or for five minutes in a Waring Blendor. The resulting homogenate was the crude enzyme.

Measurement of Catalatic Activity.—An aliquot of the crude enzyme solution, usually 5 cc., was mixed with sufficient *M*/15 phosphate buffer, pH 7.0, to make a total of 48 cc. of solution in a 29 x 200 mm. test tube. The resulting solution was cooled to 0° in a crushed ice-water bath (Dewar flask). A 2-cc. portion of 0.25 *N* hydrogen peroxide, also at 0°, was blown from a volumetric pipet into the reaction mixture, the stop watch started, and the solution thoroughly mixed. Five 5-cc. aliquots of the reaction mixture were withdrawn in rapid succession over a period of two to three minutes and added to 5-cc. portions of 2 *N* sulfuric acid. The time at which the meniscus passed an etched line on the pipet bulb was recorded for each sample. The hydrogen peroxide remaining at each time was determined by adding 10 cc. of 10% potassium iodide and a drop of 1% ammonium molybdate to the acidified samples, and, after three minutes, titrating the liberated iodine to a starch endpoint with 0.005 *N* sodium thiosulfate.

Attempts to titrate residual hydrogen peroxide with potassium permanganate were unsuccessful due to consumption of the oxidant by cellular debris. However, the iodometric procedure (5) gave satisfactory results. The allowable variation in the time of titration of liberated iodine following addition of potassium iodide to the acidified samples was tested. Reduction of hydrogen peroxide was complete after two minutes, but a gradual evolution of iodine, due to air oxidation of iodide ion, continued. Therefore, titrations were routinely made between three and five minutes after addition of the potassium iodide.

Calculation of Results.—No deviations from a first order disappearance of hydrogen peroxide were observed. In every case a plot of the common logarithm of the sodium thiosulfate titer *vs.* time of quenching of the enzymatic reaction in acid gave a straight line. The exact normalities of the reagents involved were not required. Typical rate curves are shown in Fig. 1. The first order pseudo constant, *k*, the slope of the rate curve, was directly proportional to the catalase content as indicated in Table I. Therefore, results have been expressed in terms of *k* evaluated under the conditions described above. Actually, *k* is the product of the specific reaction rate constant and the catalase concentration.

Variation in Catalase Content with Growth of the Microorganism.—A typical experiment in which variation in catalase content with growth of *C. blakesleeana* H-334 was evaluated is presented. A total of 400 cc. of seed grown sixty-six hours in

* Received May 31, 1957, from the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.
The author is indebted to E. J. Collins for the nitrogen determinations.

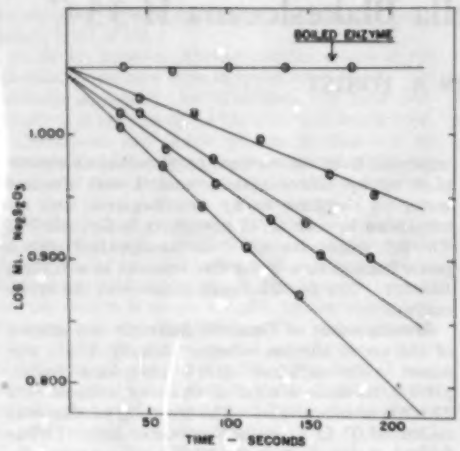

Fig. 1.—Typical rate curves.

TABLE I.—RELATIONSHIP OF PSEUDO CONSTANT TO CATALASE CONCENTRATION

Homogenate (cc.)	$k \times 10^3$(sec^{-1})	k/cc. $\times 10^3$(sec^{-1})
2	51.0	25.5
3	80.3	26.8
4	91.0	22.8
5	123.0	24.6
		Av. 24.9 ± 0.9

shake flasks on Czapek-Dox solution with 0.3% added aspartic acid was added to a 12 x 12 inch battery jar fermenter containing 8 L. of Czapek-Dox solution with 0.1% added aspartic acid. The mixture was agitated at 275 r. p. m. and aerated at 0.5 L. of air per minute at 29–32°. Periodically, aliquots of the cell suspension were removed and the catalase content determined as outlined above. Nitrogen content of the cells was determined by a micro-Kjeldahl procedure.

RESULTS AND DISCUSSION

Preparation of Crude Enzyme.—Results of a series of determinations designed to indicate the best method of preparation of the crude catalase are shown in Table II. From these data it can be concluded that (*a*) little activity is measurable without some type of cell rupture, (*b*) homogenization in distilled water or buffer is equally effective, (*c*) buffer tends to extract more of the enzyme into solution, and (*d*) pestle homogenization following treatment in a Waring Blendor does not liberate additional enzyme. In routine determinations, pestle homogenization was selected because the resulting solutions were easier to pipet, and buffer was employed since Svendsen (6) has suggested that the presence of salts lowers catalase loss due to adsorption on glass surfaces.

Properties of the Crude Catalase.—Activity of the crude catalase from *C. blakesleeana* H-334 is independent of pH over the range 6.2–7.7. The crude enzyme is stable for at least two days when stored under refrigeration. Catalatic activity is

TABLE II.—VARIATION IN ACTIVITY OF CRUDE CATALASE WITH METHOD OF PREPARATION

Expt.	Preparation	$k \times 10^3$(sec^{-1})
1a	Whole cells	8
1b	Homogenate of 1a (Waring Blendor 3 min.)	59
2a	Cell homogenate (Waring Blendor 5 min., distilled water)	67
2b	Cell homogenate (Waring Blendor 5 min., phosphate buffer)	70
2c	Supernatant from 2a	2
2d	Supernatant from 2b	28
2e	Precipitate from 2b	42
2f	2e resuspended and pestle homogenized	45

destroyed by boiling (Fig. 1) and is inhibited by 2,4-dichlorophenol (7). Electron bombardment of whole cells (2,000,000 r. e. p.–35 seconds) does not reduce the catalase content.

Variation in Catalase Concentration with the Growth of C. Blakesleeana H-334.—The change in catalase concentration during the growth of *C. blakesleeana* H-334 on a modified Czapek-Dox medium is shown in Fig. 2 along with the change in

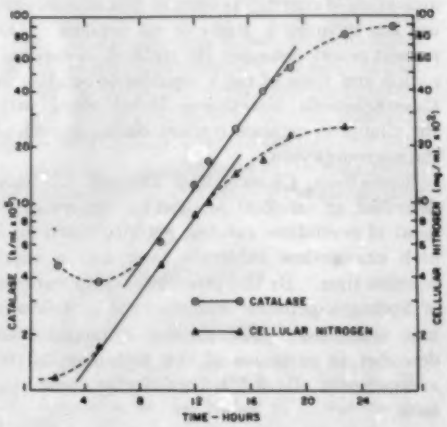

Fig. 2.—Variation in catalase content and cellular nitrogen during growth of *C. blakesleeana* H-334 on a synthetic medium (Czapek-Dox solution plus 0.1% aspartic acid).

cellular nitrogen. Catalase concentration follows a typical S-shaped growth curve. During the logarithmic phase, both catalase and cellular nitrogen increase at approximately the same rate. However, this logarithmic phase begins considerably later in the case of catalase and continues for some time after cellular nitrogen has begun to plateau. Apparently the older, mature cells are the catalase producers. The higher catalase concentration early in the growth period is believed due to the presence of the mature, catalase-rich cells from the seed. Nielson (8) has reported that young cells of *C. blakesleeana* H-334 are the most active in the conversion of 11-desoxy-17α-hydroxycorticosterone to

17α-hydroxycorticosterone. It therefore appears that catalase is not involved in this transformation.

SUMMARY

The technique of Bonnichsen, Chance, and Theorell (2) has been extended to catalase measurements in crude cell homogenates. Properties of the catalase of *Cunninghamella blakesleeana*, strain H-334, have been examined. Variation in catalase content of cells during the growth of the microorganism has been determined.

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Investigation of Human Skin Lipids I*

By JAMES E. TINGSTAD, DALE E. WURSTER, and TAKERU HIGUCHI

A method of harvesting relatively large quantities of skin lipids from human volunteers is presented. Experimental evidence indicate that the collected lipids are composed mainly of those produced by the sebaceous glands and that the lipids obtained from the arm and back regions of the body are similar in chemical composition. No significant chemical decomposition of the skin lipids was detected when the material was stored at low temperatures. The chemical species which are responsible for observed seasonal variations in the composition of human skin lipids have been determined.

THE MECHANISM whereby drugs penetrate the skin following external application has been under investigation for several decades. It is generally accepted that three possible routes of penetration exist: (1) along ducts of eccrine sweat glands into the glands and then into the surrounding tissue fluid, (2) directly through the layers of skin, and (3) down hair follicles and sebaceous ducts, then through the sebaceous glands and into the surrounding tissue fluid.

Both Rothman (1) and MacKee (2) have indicated the importance of the follicular pathway in the penetration of skin by drugs. In later work, MacKee, *et al.* (3), working with dyes, sulfas, and heavy metals, indicated that most of the penetration by these agents occurred via the follicular route. More recently it has been stated (4) that perifollicular whealing follows the application of histamine to the skin. The latter strongly suggests that transfollicular penetration occurs.

If the follicular route is an important one in the absorption of drugs through the skin, it is likely that skin lipids, both in the lipid surface film and in the follicles, play an important part in determining speed and extent of absorption. It would thus be desirable, as an initial step in a broad study of skin penetration, to determine the behavior of drugs in skin lipids. In order to make these extensive *in vitro* studies, adequate amounts of human skin lipids must first be collected.

This paper presents (*a*) a method of harvesting skin lipids from human volunteers on a large scale; (*b*) a study of the variations in the chemical composition of the collected material as a function of the method employed, the time of the year, and the body area extracted; and (*c*) the results of an investigation of the chemical nature of the components responsible for the observed variation.

EXPERIMENTAL

Collection of Skin Lipids.—The skin lipids were obtained from human volunteers by means of direct extraction from the arms with a volatile solvent.

Mass lipid extractions were made at irregular intervals over a period of nearly two years, during which time the skin lipids from the arms of more than 1,000 student volunteers were obtained. Each

* Received May 1, 1957, from the laboratories of the School of Pharmacy, University of Wisconsin, Madison.
Presented to the Scientific Section, A. PH. A., New York meeting, May 1957.
This work was completed under United States Army Chemical Corps Contract No. DA-18108-CML-5753.
Based on a dissertation submitted by James E. Tingstad to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

mass extraction contained the pooled lipids of approximately 50 subjects.

Solvent.—Ethyl ether (reagent grade), which was distilled just before use to avoid the dangers associated with peroxide formation, was used as the extracting medium.

Collection Procedure.—The hands and arms of each subject were washed with a sodium lauryl sulfate solution, rinsed with distilled water, and dried. The hands were then immersed in ether for thirty seconds to insure complete removal of contaminants from this area. This portion of the ether wash was discarded.

The hand and arm were then immersed in the purified ether (1,750 ml.) contained in a glass tube 100 mm. in diameter and 56 cm. high. A wooden block was used to hold the extraction tube in place. The arm was maintained in the tube for one minute; the procedure was then repeated on the other arm. The ether in the tube was replaced after 15 subjects had been subjected to the extraction procedure.

The lipids were recovered from the ether solution by distilling off the solvent. When the volume was sufficiently reduced (150 ml.), the concentrated lipid solution was filtered through a medium-porosity, sintered glass filter. The clear filtrate was then carefully heated, with agitation, under a stream of nitrogen to remove all traces of solvent.

Analysis of the Lipids.—The gross chemical composition of each sample of collected skin lipids was determined by infrared analysis and by acid and iodine number determinations. Infrared spectra were obtained on a Baird Associates infrared spectrophotometer, employing a sodium chloride prism and carbon tetrachloride as the solvent. Acid numbers were determined by the U. S. P. XV method, except that 0.01 *N* potassium hydroxide was used as the standard base. Iodine numbers were determined by the method of Yasuda (5), except that 0.2 *N* pyridine sulfate dibromide solution was used as the halogenating agent.

Effect of Extent of Extraction on Composition.—It was desirable to determine whether the extracted lipids were essentially those being produced by the sebaceous gland. For this reason lipids from the arms of four subjects were extracted in the usual manner. After a two-hour interval, during which time great care was taken to avoid contamination of the hands or arms, the arms were again immersed in a new supply of ether for two and one-half minutes. The extracts were then analyzed chemically and by infrared.

For another series of experiments, the arms of an individual were extracted by the standard procedure at two-day intervals for a period of six days. The material collected was then subjected to infrared analysis and acid number determination.

Lipids from the Arm and the Back.—In order to determine whether the lipid samples obtained from the arm were similar to the skin lipids from other parts of the body, both spectrophotometric and chemical comparison of the lipids from the arm were made with lipid samples from the back. Since extraction of lipids from the back by immersion was not possible, a modified procedure was followed.

The back of each subject was washed and dried in the usual manner. The subjects were then placed in a prone position, and glass cylinders 3.8 cm. in diameter, 10 cm. high, and open at both ends were then held firmly against the skin of the back. About 75 ml. of ether was then placed in the cylinder and the cylinder was carefully moved over the skin surface. The time of exposure to the solvent for each area was approximately fifteen seconds. After the extraction period, the lipid solution was removed from the cylinder with a pipet. The lipids were then recovered in the previously described manner. Extraction from the arms of the subject from the elbows to the shoulders was made by the same procedure. The gross composition of the two lipid samples was determined in the usual manner.

Deterioration During Storage.—To determine the effects of storage on skin lipids, two portions of a sample of lipids were stored under different conditions: one in a refrigerator (5–10°), and the other in a Dewar flask filled with dry ice (−80°). Chemical and spectral analyses were performed at the end of six and fifteen weeks.

Chemical Composition of Skin Lipids.—Although considerable research effort has already been directed toward the determination of skin lipid composition by others (6), it was necessary to establish the chemical nature of the component or components responsible for the observed variations in lipid composition. A liquid-liquid countercurrent extraction technique (7, 8) was therefore employed to fractionate the skin lipids.

Approximately one gram of the lipids was dissolved in petroleum ether (b. p. 35–38°) and subjected to 100 transfer stages in an all-glass, Craig-type extractor (10/10). Petroleum ether was used as the moving phase and methanol containing 2½% (v/v) water as the stationary phase. After the 100th transfer, the contents of each tube were transferred to a tared aluminum moisture pan and allowed to evaporate to dryness. The solid content of each pan was then determined and infrared analyses were done on fractions of interest.

RESULTS AND DISCUSSION

Collection of Skin Lipids.—By using the described extraction procedure, it was possible to make extractions from the arms of approximately 60 to 70 people in three hours. The yield averaged 0.1 Gm. per person; a single mass extraction thus supplied about 6–7 Gm. of material. In all, over 100 Gm. of skin lipids were collected. The lipids were light brown in color and had a semisolid consistency at room temperature.

Seasonal Variation in Lipid Composition.—Infrared analysis of each of the lots of lipids indicates a possible seasonal variation in composition. Infrared spectra of typical lipid lots are shown in Figs. 1–3. The tracings are of little value in determining the absolute chemical composition of the gross mixture; however, they are useful both in establishing the relative constancy of each mixture and in detecting large variations in the amounts of different functional groups present.

Close examination of a large number of these tracings revealed that although successive samples collected over a relatively short period of time (one to two months) were quite similar, there occurred throughout the year a considerable variation in the relative amounts of the materials causing the 5.75-micron and the 5.85-micron absorption peaks. In certain lots the absorbance at 5.75 microns was greater; in others the two absorbances were about

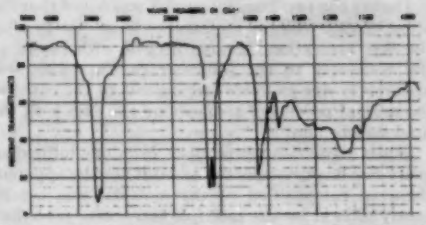

Fig. 1.—Typical infrared spectrum of lipids collected in February, March, and April, 1955.

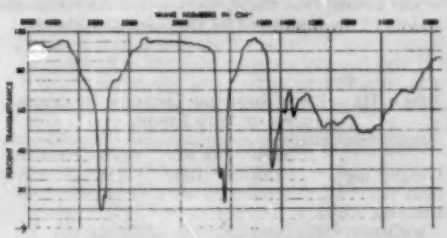

Fig. 2.—Typical infrared spectrum of lipids collected in August, September, and October, 1955.

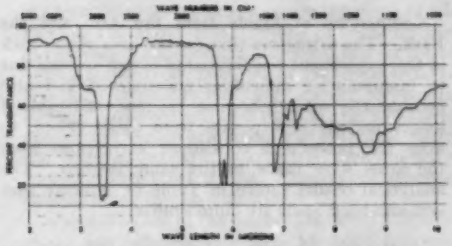

Fig. 3.—Typical infrared spectrum of lipids collected in January and February, 1956.

equal; in still others the absorbance at 5.85 microns was greater.

These variations are apparent in the data shown in Table I where the ratios of the absorbances $A_{5.75}/A_{5.85}$ for 25 different lots of skin lipids ranging from a low of 0.62 to 1.06 are given. The seasonal dependency of this ratio as indicated by the table is more apparent in Fig. 4 where it has been plotted against the time of collection.

The data suggest that the relative concentrations of the substances causing the 5.75-micron peak reach a maximum during late winter and early spring and a minimum during the months of August and September—the 5.85-micron substance following a converse pattern. This pattern of annual rise and fall is manifested during both years encompassed in the present study. This biochemical response may possibly be due to changes in the thermal environment, the diet of the volunteers during the course of the year, the presence of lipases elaborated by microorganisms, or a combination of these factors.

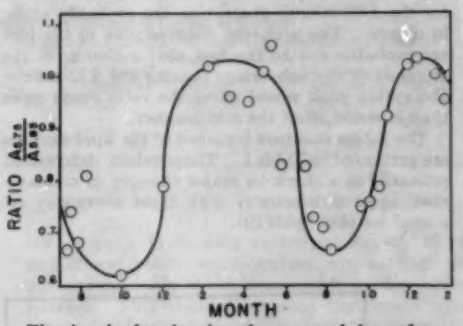

Fig. 4.—A plot showing the seasonal dependency of the absorbance ratio of the 5.75 micron to the 5.85 micron peak in the infrared (lipids from mass collections). See Table I.

Since each sample represented total lipids collected from about 50 subjects, it was believed that the effects of variations among individuals were not reflected in the data shown. This assumption was verified by work done later on individuals. Results of that study will be presented in a subsequent paper.

Acid numbers obtained for some of the lipid samples are presented in Table I. Analyses were not made on every sample, since some lots were collected specifically for other studies. In this table the acid numbers of the lipid samples subjected to analysis appear to vary with the infrared absorbance

TABLE I.—RESULTS OF ANALYSIS OF MASS EXTRACTIONS

Date	Infrared Peak Ratio, $A_{5.75}/A_{5.85}$	Acid No.	Iodine No.
7/6/54			
7/10	0.67		
7/16	0.74	73	52
7/28	0.68	93	60
8/9	0.81	78	54
10/1	0.62	109	54
12/1	0.79	61	60
2/9/55	1.02	49	68
3/9	0.96	56	60
4/6	0.95	62	69
4/28	1.01	..	..
5/11	1.06	..	..
5/25	0.83	..	..
6/29	0.83	77	50
7/13	0.73	88	42
7/27	0.71	98	43
8/10	0.67	82	52
9/21	0.75	..	..
10/5	0.76	..	..
10/19	0.79	..	..
11/2	0.92	..	..
11/30	1.02	..	..
12/14	1.03	..	..
1/11/56	1.00	..	..
1/25	0.95	..	..
2/8	1.00	..	..

ratio discussed above. This is more evident in Fig. 5 where the acid number has been plotted against the reciprocal of the ratio. The data suggest that the component or components responsible

for the 5.85-micron absorption are probably acidic in nature. The apparent discrepancies in the plot are probably due to the fact that a change in the amounts of the substances causing the 5.75-micron absorption peak would affect the ratio much more than it would affect the acid number.

The iodine numbers for some of the lipid samples are presented in Table I. These values, determined primarily as a check on major changes in composition, agree satisfactorily with those previously reported for skin lipids (9).

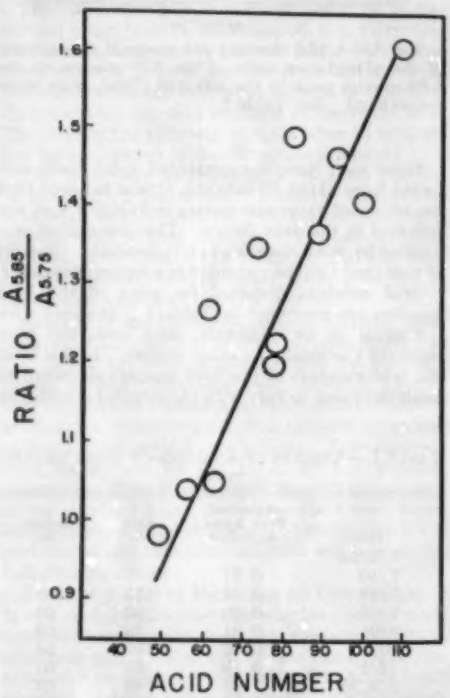

Fig. 5.—A plot showing the dependency of acid numbers on the absorbance ratio. See Table I.

Effect of Extent of Extraction on Composition.—Results of analyses on the material obtained from extractions performed at a two-hour interval are given in Table II. The data suggest that very little difference exists between the two lots.

TABLE II.—EFFECT OF SUCCESSIVE EXTRACTIONS ON COMPOSITION

Sample	Wt., Gm.	Iodine No.	Acid No.	Infrared Ratio of Absorbance, $A_{5.75}/A_{5.85}$	
First Extract	0.400	76	49	1.12	1.10[a]
Second Extract	0.020	70	59	1.09	1.07[a]

[a] These are the results for duplicate extractions on the same individuals.

During the two-hour interval it was noted that the arms, chalky white in appearance immediately after the first extraction, became flecked with brown at the entrances to the follicular canals. This was apparently due to lipids seeping out of the canal in the normal process of resupplying the skin with the lipid surface film.

When extractions were made from one individual every two days, neither the amount nor the apparent chemical composition of the material was significantly altered by the multiple extraction procedure (see Table III). This strongly suggests that the skin lipids are fully regenerated in 48 hours at least. It would also appear, especially in view of the fact that the lipids were observed seeping out of the follicular canals, that the lipids collected are composed mainly of those secreted by the sebaceous glands.

TABLE III.—DATA SHOWING UNIFORM REGENERATION OF SKIN LIPIDS

	Initial	48 Hr.	96 Hr.	144 Hr.
Weight, mg.	135	150	118	155
Acid No.	50	46	46	42
Infrared Absorbance Ratio, $\frac{A_{5.75}}{A_{5.85}}$	1.25	1.04	1.07	1.20

Comparison of Lipids from the Arm and the Back.—The extraction time for the area of the back in this experiment was limited to fifteen seconds due to manipulative difficulties encountered and also because the subject became too uncomfortable if longer periods of exposure were used. Then, to keep the procedures consistent, extractions from the arms were made in the same manner. The analytical results shown in Table IV indicate that arm and back lipids are quite similar.

TABLE IV.—COMPARISON OF BACK AND ARM SKIN LIPIDS

	Arms (1)	Back (1)	Arms (2)	Back (2)
Acid No.	39	39	[a]	[a]
Iodine No.	75	87	[a]	[a]
Infrared Absorbance Ratio, $\frac{A_{5.75}}{A_{5.85}}$	1.54	1.34	1.65	1.85

[a] Amount of material collected here was too small for chemical analysis.

Deterioration During Storage.—From the experimental data obtained, it is apparent that the extracted lipids can be stored for extended periods without undergoing significant chemical changes. Results of the various analyses are given in Table V.

The initial iodine number is somewhat lower than the others, but no significant differences exist between the stored lots and the original sample. It thus appears that the lipids can be stored for long periods of time if kept at cold temperatures.

TABLE V.—DATA SHOWING EFFECTS OF STORAGE ON SKIN LIPIDS

		Storage Time			
	Original	6 Weeks Dry Ice	Refrig.	15 Weeks Dry Ice	Refrig.
Acid Number	56	56	56	56	56
Iodine Number	60	71	70	69	71
Infrared Absorbance Ratio, $A_{5.75}/A_{5.85}$	0.94	0.98	0.98	1.01	1.00

Chemical Composition of Skin Lipids.—In Fig. 6 a typical distribution pattern obtained by the countercurrent extraction procedure is shown. It was evident from infrared analyses that nearly complete separation of the materials causing the two absorption peaks was accomplished.

The material found in Tubes 40–42 was crystalline in appearance, strongly absorbed infrared light in the 5.85-micron region, and had an acid number of 180. Further countercurrent extractions were performed on this fraction in an effort to purify it. Although only one peak continued to appear in the chromatograms, successive extractions did help to purify the material. Infrared tracings of the purified material indicated that it was composed largely of fatty acids of the palmitic, stearic, and arachidic type. A neutral equivalent determination resulted in an equivalent weight of 306, which is in the range of the mixtures of C_{16} to C_{20} fatty acids previously reported (10). Furthermore, melting point determinations on various fractions resulted in melting points ranging from 50 to 60°, which is also in the range of mixtures of the above acids. None of these fractions gave a positive Lieberman-Burchard test for sterols.

The material in Tube 90 of the countercurrent transfer operation did not appear crystalline, strongly absorbed infrared light in the 5.75-micron region, and had an acid number of only five. Infrared tracings indicated that esters were the major components of this fraction. This was subsequently substantiated by saponification.

About 0.5 Gm. of the material believed to contain esters was refluxed with 20% alcoholic potassium hydroxide for nine hours. After extraction and purification, the nonsaponifiable portion did not absorb in the 5.75-micron region; while the 5.85-micron peak (indicating carboxylic acids), which was absent before saponification, appeared in the infrared spectrum of the base-soluble portion of the mixture. This definitely showed that esters were responsible for the 5.75-micron absorption peak.

The material in Tube 95 of the original countercurrent transfer operation gave a positive Lieberman-Burchard test, indicating that sterols were also present in this fraction. More work is currently being done in an effort to further fractionate the nonacid portion of the skin lipids.

These data indicate that the chemical species responsible for the observed seasonal variations in human skin lipids are free fatty acids and esters of fatty acids, since it has now been definitely established that these chemical entities are responsible for the observed absorbance peaks at 5.85 and 5.75 microns, respectively, in the infrared tracings.

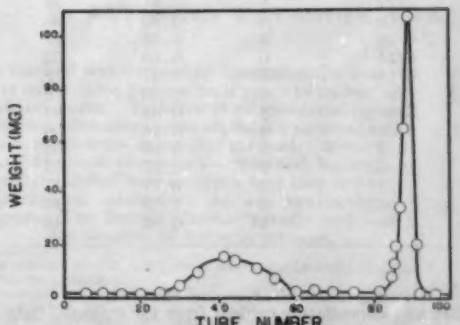

Fig. 6.—A plot showing the distribution of human skin lipids between petroleum ether and 97.5% methanol (100 transfers).

SUMMARY

A procedure for extracting relatively large quantities of human skin lipids is presented. The lipids collected were found to vary significantly with the season of the year. Successive extractions within a short time (two hours and also two days) did not alter the composition of the lipids, nor did extractions performed every two days alter the amount of lipids collected. The lipids from the arms were found to be quite similar to those obtained from the back. The lipids were found to be quite stable if stored at temperatures below 10°. A countercurrent extraction procedure for the fractionation of skin lipids is presented. Fatty carboxylic acids and esters of those acids, which absorb in the 5.85 and 5.75-micron regions, respectively, of the infrared spectrum, are deemed responsible for the observed seasonal variations in human skin lipid composition.

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Investigation of Human Skin Lipids II*

By JAMES E. TINGSTAD, DALE E. WURSTER, and TAKERU HIGUCHI

Skin lipids from different individuals vary considerably in chemical composition. The seasonal variation in skin lipid composition suggested in a previous report has been confirmed. The nonsaponifiable fraction of skin lipids can be further fractionated using a liquid-liquid countercurrent extraction procedure.

In a recent report from these laboratories (1) a possible seasonal variation in the chemical composition of human skin lipids was suggested. However, since the results were obtained on samples containing the pooled lipids of a large number of persons, the possibility existed that the observed variations were artifacts resulting from variations among the skin lipids of the various individuals from whom the lipids were obtained. Therefore, this study was initiated in an attempt to verify the seasonal variation phenomenon and to ascertain the differences in skin lipid composition among individuals.

It was previously reported (1) that skin lipids could be fractionated using a liquid-liquid countercurrent extraction procedure. This resulted in an acid and a nonacid portion, the latter consisting mostly of wax alcohols, sterols, esters, and hydrocarbons. The saponification of the esters produced free fatty acids and a nonsaponifiable fraction consisting mainly of alcohols and hydrocarbons. In this report, results of an attempt to further fractionate this nonsaponifiable fraction are presented.

EXPERIMENTAL

Variations in Skin Lipid Composition Among Individuals.—The arm lipids from ten individuals were obtained in the manner previously described (1). Extractions were made every four weeks for a period of fifteen months. The lipid samples obtained from each individual were treated separately. The gross composition of each sample was followed with infrared analysis and acid number determinations.

Fractionation of the Nonsaponifiable Fraction of Skin Lipids.—A sample (400 mg.) of the nonsaponifiable fraction of the skin lipid material was dissolved in petroleum ether (b. p. 35–38°) and subjected to 100 transfer stages in an all-glass, Craig-type extractor (10/10). Petroleum ether was used as the moving phase and methanol containing $2^1/_2$% (v/v) water as the stationary phase. After the 100th transfer, the contents of each tube were transferred to a tared aluminum moisture pan and allowed to evaporate to dryness. The solid content of each pan was then determined and infrared analyses were performed on the fractions of interest.

RESULTS AND DISCUSSION

Seasonal Variations in Skin Lipid Composition Among Individuals.—As previously reported (1) the absorbance ratio, $A_{5.75}/A_{5.85}$, obtained from infrared studies on pooled human skin lipids, where the 5.75-micron peak represents esters and the 5.85-micron peak represents carboxylic acids, showed a marked seasonal variation. The absorbance ratios obtained for the ten individuals used in this study show the same periodic fluctuation. Results of the infrared analyses and acid number determinations of lipid samples from two individuals representing the extremes of the test group are given in Table I.

Table I.—Results of Analysis of Individual Lipid Extracts Obtained from Subjects Representing Extremes of the Test Group

Date	Sample, Test Subject No.	Infrared Peak Ratio, $A_{5.75}/A_{5.85}$	Acid Number
1/10/56	3	2.03	..
2/22	3	1.19	40
3/19	3	1.42	31
4/16	3	1.65	33
5/14	3	1.23	51
6/11	3	1.25	44
7/9	3	1.02	48
8/6	3	1.06	60
9/6	3	1.14	68
10/4	3	1.48	39
10/30	3	1.24	41
11/27	3	1.62	36
1/9/57	3	1.75	32
2/8	3	1.55	34
1/10/56	6	..	..
2/22	6	0.87	54
3/19	6	0.70	50
4/16	6	0.64	57
5/14	6	0.58	52
6/11	6	0.47	72
7/9	6	0.39	73
8/6	6	0.54	64
9/6	6	0.46	66
10/4	6	0.54	58
10/30	6	0.57	59
11/27	6	0.77	57
1/9/57	6	1.03	40
2/8	6	0.81	45

It was immediately evident from the collected data that great variations in lipid composition exist among individuals; however, even though these differences do exist, the lipids of each tested subject

* Received July 11, 1957, from the laboratories of the School of Pharmacy, University of Wisconsin, Madison.
This work was completed under United States Army Chemical Corps Contract No. DA-18108-CML-5753.
Based on a dissertation submitted by James E. Tingstad to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

followed the same general pattern as far as the seasonal variation is concerned. This is particularly evident when the data is plotted as in Fig. 1. Here it can be observed that the peaks and valleys in this plot occur at the same time of the year as they did in a similar plot for the lipids collected in the previously reported mass extractions (1). The same phenomenon was observed in all the members of the test group. This strongly indicates that a seasonal variation in the skin lipid composition of individuals does occur in this climate.

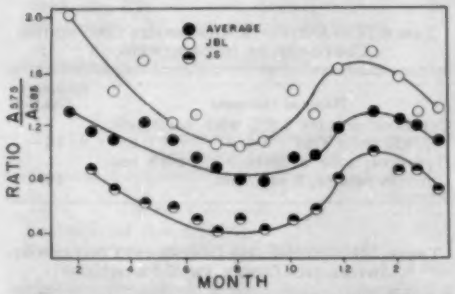

Fig. 1.—A plot showing the seasonal dependency of the absorbance ratio of the 5.75-micron (esters) to the 5.85-micron peak (carboxylic acids) in the infrared. (● Average for the 10 individuals tested, ○ JBL—test subject no. 3, ◓ JS—test subject no. 6 See Table I.)

Fraction of the Nonsaponifiable Fraction of Skin Lipids.—The separation of the nonsaponifiable fraction of skin lipids into two fractions is shown in Fig. 2. Infrared tracings showed that the first fraction contained appreciable amounts of alcohols and the second fraction contained mostly hydrocarbons. This was further indicated by the fact

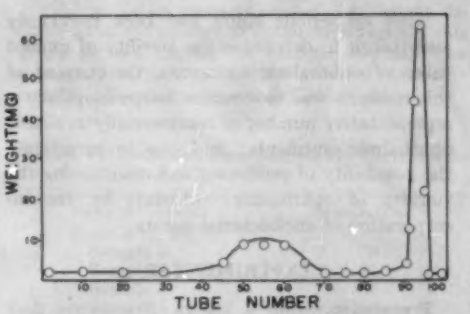

Fig. 2.—A plot showing the distribution of the nonsaponifiable fraction of human skin lipids between petroleum ether and 97.5% methanol (100 transfers).

that the first fraction gave a positive Lieberman-Burchard test for sterols, while the second did not. Thus it appears that the liquid-liquid countercurrent extraction procedure is a suitable method for the fractionation of human skin lipids.

SUMMARY

A considerable variation in the chemical composition of skin lipids exists among individuals. A seasonal variation in skin-lipid composition, which appears to be common to most people in this climate, was demonstrated in ten individuals. A procedure for the fractionation of the nonsaponifiable fraction of human skin lipids is presented.

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A Bacteriological Study of Ophthalmic Ointments*

By RAYMOND W. VANDER WYK† and ANDREW E. GRANSTON

A method was devised to determine the number of bacteria present in a gram of ophthalmic ointment. Eighty-three commercial ophthalmic ointments were tested for contamination by bacteria using this method. Seventy-one of these ointments contained bacteria. Benzyl alcohol was incorporated into several ophthalmic ointments as an antibacterial agent. The benzyl alcohol greatly reduced the number of bacteria present.

* Received May 3, 1957, from the Massachusetts College of Pharmacy, Boston.
Presented to the Scientific Section, A. PH. A., New York meeting, April, 1957.
This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Andrew E. Granston in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
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IN RECENT YEARS considerable attention has been directed toward the desirability of producing and maintaining the sterility of liquid ophthalmic preparations (1). Several workers have described methods for sterilization including the use of heat (2), the use of chemical antibacterial agents (3), and the use of bacterial excluding filters (4).

However, a survey of the literature has revealed that very little work has been published concerning the testing for sterility of ophthalmic ointments. Some research was done by Lehrfeld and Donnelly (5), who tested for the most part the sterility of partly used tubes of ointment.

Since no serious study had been previously undertaken to determine the sterility of unused tubes of ophthalmic ointments, the purpose of this research was to examine bacteriologically a representative number of commercially available ophthalmic ointments; and also to investigate the possibility of producing and maintaining the sterility of ophthalmic ointments by the incorporation of antibacterial agents.

EXPERIMENTAL

Preparation.—Into a 250-ml. Erlenmeyer flask was poured 25.0 ml. of distilled water and a small number of 0.5-mm. glass beads. Then the flask was plugged and sterilized in an autoclave.

Testing.—Each ointment to be tested was immersed for one hour in its unopened tube in a 1:1,000 solution of benzalkonium chloride in order to kill any bacteria present on the surface of the tube.

The tube was removed from the benzalkonium chloride solution, the lip of the flask and the tip of the tube were flamed simultaneously, and the contents of the tube were squeezed into the flask.

Then the flask was warmed in a constant-temperature water bath to 45°. At this temperature the ointment melted and was easily dispersed throughout the water by being shaken on an agitator for one hour at room temperature.

At the end of the agitation period, three 1.0-ml. portions were transferred with a sterile pipet into three sterile Petri dishes. To these was added melted blood agar in the usual manner for preparing pour plates. After mixture by gentle rotation and after solidification of the agar, the plates were incubated for twenty-four hours at 37°.

Determination of the Number of Bacteria per Gram.—The number of bacteria in 1 Gm. of each sample was determined by counting the colonies on the plate. Each colony was assumed to represent one organism in the ointment. The following formula was used in this determination:

$$\text{No. of bacteria per Gm.} = \frac{(\text{No. of colonies per plate} \times 25)}{\text{(Wt. of contents of the tube)}}$$

In all instances the weight of the contents of the tube was 3.5 Gm.

Results.—Of the 83 commercially available ophthalmic ointments tested 12, or 14.5%, were sterile. However, only 19 of the 71 nonsterile ointments had 50 or more organisms per gram.

For convenience in discussing the results of this work the ointments have been classified according to their principal chemotherapeutic agents.

Antibiotic Ointments.—Twenty-eight were tested and only eleven were sterile. However, the bacterial count was low, the average bacterial count being only 12 organisms per gram. The results appear in Tables I, II, and III.

Sulfa-Containing Ointments.—Nine were tested, and only one was sterile. However, the average bacterial count was only 15 organisms per gram. The results are shown in Table IV.

Mercury-Containing Ointments.—Twenty-one ointments were tested, and none was found to be sterile. The average number of bacteria per gram for these ointments was 44. The results are shown in Table V.

Cortisone and Hydrocortisone Ointments.—Four ointments were tested and none was sterile. All showed identical bacterial counts of 14 bacteria per gram. This low count is very surprising in view of the fact that none of the ointments tested contained anti-infective agents. The results appear in Table VI.

Boric Acid Ointments.—Six were tested and none was found to be sterile. The average number of

TABLE I.—PENICILLIN OPHTHALMIC OINTMENTS

Name of Ointment	Bacteria/ Gm.
Crystalline procaine penicillin G, 5,000 units/Gm.	14
Penicillin calcium, 100,000 units/Gm.	0
Penicillin G, 1,000 units/Gm.	
Sample 1	7
Sample 2	14
Penicillin G, 100,000 units/Gm.	7
Penicillin G potassium, 1,000 units/Gm.	0
Procaine penicillin G, 1,000 units/Gm.	64

TABLE II.—ANTIBIOTIC OINTMENTS CONTAINING CORTISONE OR HYDROCORTISONE

Name of Ointment	Bacteria/ Gm.
Cortisone acetate 1.5% with bacitracin, 1,000 units/Gm.	14
Hydrocortisone acetate 0.5% with neomycin sulfate, 5 mg./Gm.	14

TABLE III.—ANTIBIOTIC OINTMENTS CONTAINING ANTIBIOTICS OTHER THAN PENICILLIN

Name of Ointment	Bacteria/ Gm.
Bacitracin, 500 units/Gm.	
Sample 1	0
Sample 2	0
Sample 3	7
Sample 4	7
Sample 5	0
Chloramphenicol, 1%	0
Chlortetracycline hydrochloride, 1%	0
Erythromycin, 5 mg./Gm.	
Sample 1	129
Sample 2	7
Neomycin sulfate, 5%	
Sample 1	7
Sample 2	0
Neomycin hydrochloride, 2.5 mg./Gm., and gramicidin, 0.25 mg./Gm.	7
Neomycin sulfate, 5 mg./Gm., polymyxin B sulfate, 5,000 units/Gm., and bacitracin, 400 units/Gm.	
Sample 1	0
Sample 2	14
Oxytetracycline hydrochloride, 5 mg./Gm., with polymyxin B sulfate, 10,000 units/Gm.	0
Polymyxin B sulfate, 20,000 units/Gm.	0
Tetracycline hydrochloride, 1%	
Sample 1	7
Sample 2	14
Sample 3	7

TABLE IV.—OPHTHALMIC OINTMENTS CONTAINING SULFA DRUGS

Name of Ointment	Bacteria/Gm.
Sodium sulfacetamide, 10%	14
Sulfadiazine, 5%	
Sample 1	7
Sample 2	21
Sulfanilamide, 5%, phenacaine hydrochloride, 1%, and cod liver oil concentrate	21
Sulfathiazole, 5%	
Sample 1	7
Sample 2	0
Sulfathiazole, 5%, and phenacaine hydrochloride, 2%	7
Sulfathiazole, 5%, and piperocaine hydrochloride, 4%	36
Sulfisoxazole diethanolamine, 4%	21

TABLE V.—OPHTHALMIC OINTMENTS CONTAINING MERCURY COMPOUNDS

Name of Ointment	Bacteria/Gm.
Ammoniated mercury, 3%	107
Butacaine sulfate, 2%, and nitromersol, 1:3,000	29
Mercuric chloride, 1:3,333	14
Mercuric chloride, 1:5,000	
Sample 1	21
Sample 2	50
Phenacaine hydrochloride, 2%, with mercarbolide	21
Phenylmercuric nitrate, 1:30,000	14
Phenylmercuric nitrate, 1:20,000, and phenacaine hydrochloride, 1%	36
Piperocaine hydrochloride, 4%, and thiomersal, 1:5,000	36
Thiomersal, 1:5,000	64
Yellow mercuric oxide, 1%	
Sample 1	64
Sample 2	14
Sample 3	50
Sample 4	57
Sample 5	43
Yellow mercuric oxide, 2%	
Sample 1	14
Sample 2	121
Sample 3	43
Sample 4	36
Sample 5	50
Sample 6	36

TABLE VI.—OPHTHALMIC OINTMENTS CONTAINING CORTISONE OR HYDROCORTISONE

Name of Ointment	Bacteria/Gm.
Cortisone acetate, 1.5%	
Sample 1	14
Sample 2	14
Hydrocortisone acetate, 1.5%	
Sample 1	14
Sample 2	14

bacteria per gram was 55. The results are shown in Table VII.

Miscellaneous Ointments.—This group consisted of ophthalmic ointments, with the exception of cortisone, which are used for purposes other than anti-infective.

Fifteen ointments were tested. As might be expected in the absence of antibacterial agents, this group showed the highest bacterial counts. The average bacterial count for this group of ointments was 65. The results appear in Table VIII.

TABLE VII.—OPHTHALMIC OINTMENTS CONTAINING BORIC ACID

Name of Ointment	Bacteria/Gm.
Boric acid, 5%	
Sample 1	79
Sample 2	21
Sample 3	57
Sample 4	14
Boric acid, 10%	
Sample 1	136
Sample 2	21

TABLE VIII.—MISCELLANEOUS OPHTHALMIC OINTMENTS

Name of Ointment	Bacteria/Gm.
Atropine sulfate, 1%	
Sample 1	21
Sample 2	129
Butacaine sulfate, 1%	7
Butacaine sulfate, 2%	7
Cod liver oil concentrate	43
Phenacaine hydrochloride, 1%	
Sample 1	86
Sample 2	50
Phenacaine hydrochloride, 1%, and ephedrine hydrochloride, 0.4%	157
Phenacaine hydrochloride, 1%, and epinepherine, 1:50,000	
Sample 1	36
Sample 2	243
Sample 3	14
Physostigmine salicylate, 0.25%	43
Piperocaine hydrochloride, 4%	107
Thenylpyramine, 0.5%	36
Zinc sulfate, 0.5%	43

OPHTHALMIC OINTMENTS CONTAINING ANTIBACTERIAL AGENTS

A Specially Formulated Ointment Base.—With few exceptions the commercial samples of ophthalmic ointments previously tested contained petrolatum in their formulas. Since petrolatum is not the ideal ointment base for topical application, a different base was decided upon. This base has the following formula:

White Wax	1 part
Cetyl Alcohol	1 part
Deltyl Extra[1]	4 parts

The white wax and the cetyl alcohol were melted in a beaker and the warm Deltyl Extra was added. The mixture was stirred until it congealed.

The ointment base contains 0.16% of water and is miscible with the the lachrymal secretions. It can be sterilized by dry heat for two hours at 175° without apparent breakdown.

[1] Deltyl Extra is manufactured by Givaudan Delawanna, Inc.

A Method for Testing the Effectiveness of Added Antibacterial Agents.—Twenty grams of ointment base were prepared and sterilized. Four 4-mm. loopfuls of a twenty-four-hour nutrient broth culture of *Micrococcus pyogenes* var. *aureus* were added and mixed thoroughly. Three and one-half grams of this contaminated base were placed in a sterile ophthalmic tube as a control.

To 9.5 Gm. of the remaining base was added 0.5 ml. of a 1:250 dilution of benzalkonium chloride. The final concentration of benzalkonium chloride was 1:5,000. The mixture was tubed in 3.5-Gm. amounts.

Within eight hours of preparation, each tube was tested to determine the number of bacteria per gram. The method is described under "Experimental."

A similar study was made with final concentrations of 0.5% chlorobutanol, 0.5% benzyl alcohol, and a mixture of 0.25% chlorobutanol and 0.25% benzyl alcohol.

In considering the effectiveness of the antibacterial agents used, it should be borne in mind that the number of organisms added to each ointment was far in excess of the number normally found in any commercial ointment. This was done purposely in order to obtain results which would be more significant. The effectiveness of each agent was based upon the number of organisms killed or inhibited as compared to a control. The results are shown in Table IX.

TABLE IX.—A COMPARISON OF THE BACTERICIDAL EFFECTIVENESS OF CERTAIN ANTIBACTERIAL AGENTS IN A CONTAMINATED OINTMENT BASE

Antibacterial Agent	No. of Organisms/Gm.	Killed, %
Benzalkonium chloride, 1:1000	835	98.0
Chlorobutanol, 0.5%	4,480	89.3
Benzyl alcohol, 0.5%	171	99.6
Chlorobutanol, 0.25%, and benzyl alcohol, 0.25%	11,409	72.7
None (control)	41,729	..

Another Method of Testing the Effectiveness of Added Antibacterial Agents.—Only benzyl alcohol 0.5% was used since this agent with a killing or inhibiting activity of 99.6% was the most effective antibacterial agent used in this study.

Six ophthalmic ointments with formulas similar to those of commercially available products were prepared and benzyl alcohol was added. The final concentration of benzyl alcohol was 0.5%. Each ointment was prepared as previously described.

Bacterial counts were taken on each extemporaneously prepared sample, and the results were compared with those previously obtained for similar types of commercial samples (see Tables I, V, VI, and VII). If similar ointments from more than one manufacturer had previously been tested, the average number of bacteria per gram was taken as the basis for comparison.

The names of the ointments were yellow mercuric oxide 1%, yellow mercuric oxide 2%, boric acid 5%, boric acid 10%, cortisone acetate 1.5%, and penicillin G potassium 1,000 units per gram.

Results.—The incorporation of benzyl alcohol 0.5% in extemporaneously prepared ointments resulted in a reduction of the bacterial count in all ointments. The actual reduction by the use of benzyl alcohol was 97.1%. (See Table X.)

TABLE X.—A COMPARISON BETWEEN EXTEMPORANEOUSLY PREPARED OPHTHALMIC OINTMENTS CONTAINING BENZYL ALCOHOL 0.5% AND COMMERCIAL OPHTHALMIC OINTMENTS

Name of Ointment	Extemporaneously Prepared Samples, Bacteria/Gm.	Commercial Samples, Av. No. Bacteria/Gm.
Yellow mercuric oxide, 1%	0	46
Yellow mercuric oxide, 2%	7	50
Boric acid, 5%	0	43
Boric acid, 10%	0	79
Cortisone acetate, 1.5%	0	14
Penicillin G potassium, 1000 units/Gm.	0	11

SUMMARY

1. Eighty-three commercial samples of ophthalmic ointments were examined bacteriologically to determine the number of organisms per gram of each. Eighty-five and one-half per cent were nonsterile.

2. A specially formulated ointment base was purposely contaminated with a culture of *Micrococcus pyogenes* var. *aureus*. To this mixture was added various antibacterial agents. These ointments were tested to determine the killing or inhibitory activity of the added antibacterial agents.

3. Benzyl alcohol 0.5 per cent was 99.6 per cent effective; benzalkonium chloride 1:5000 was 98.0 per cent effective; chlorobutanol 0.5 per cent was 89.3 per cent effective; while a mixture of chlorobutanol 0.25 per cent and benzyl alcohol 0.25 per cent was only 72.7 per cent effective.

4. Six ophthalmic ointments were prepared extemporaneously by methods similar to those used by the pharmaceutical manufacturers. Benzyl alcohol 0.5 per cent was added to each. The number of organisms per gram of each ointment was determined and was compared with the average number per gram of commercially available ointments with similar formulas. Benzyl alcohol reduced the number of bacteria per gram by 97.1 per cent.

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Toxicity Studies of Sulfur Hexafluoride (SF_6) in Pneumoperitoneum*

By HAROLD C. HODGE, W. GEORGE SWALBACH†, and HERMANN RAHN‡

The results of studies of rats and dogs maintained with pneumoperitoneum of sulfur hexafluoride, and with air for control observations, for periods of six months are reported. The toxicity found was of a sufficiently low order to warrant initial treatments of humans.

EVER SINCE THE INTRODUCTION of artificial pneumothorax and pneumoperitoneum for therapeutic reasons, the reabsorption of the injected gas has been noted. For prolonged therapy this reabsorption has been a nuisance requiring refillings from time to time. It was noted early that pure oxygen disappeared much faster than air, and today air is the common gas used. Many attempts have been made during the last fifty years to find a more suitable gas than air—a gas which would be more slowly absorbed from the cavity. Webb, *et al.* (1), found no difference between air and nitrogen. Grass and Meiners (2) employed argon which was absorbed faster than air. Helium was tried unsuccessfully by Schedtler (3). More recently helium was found to extend the time between refills four to six days in pneumoperitoneum patients (4). Comparing the absorption rates of various gases from closed body pockets (5–8), however, led to the general conclusion that of all the gases so far tested nitrogen is the slowest to diffuse and therefore is most suitable for a prolonged collapse therapy. It was, therefore, of some interest when Tenney, Carpenter, and Rahn (9) showed that the inert gas SF_6 when injected into a body cavity, not only disappeared more slowly than nitrogen, but actually produced a temporary doubling of the pneumoperitoneum volume before it slowly disappeared.

Before administering SF_6 gas to pneumoperitoneum patients, it was necessary to demonstrate the lack of toxicity of this gas. This paper presents the results of studies of rats and dogs maintained with pneumoperitoneum of SF_6, and with air for control observations, for periods of six months. Based on these observations the initial treatments of patients reported elsewhere (10) were undertaken.

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RAT STUDIES

Groups of young, albino rats, 20 males and 20 females per group, of the Rochester-Wistar strain were selected and matched approximately for average body weight. The rats were housed five to a cage in metal cages in which pans filled with shavings were used for bedding. The ration was Purina Fox Chow Meal, the drinking water was tap water; both were supplied *ad libitum*. As can be seen in Table I, the average initial body weight for the male rats ranged from 149 to 154 Gm., for the female rats from 128 to 132 Gm. The treatment plan is indicated in Table I: each experimental rat was injected with a volume of SF_6 sufficient to distend the skin tightly; each control rat was injected with an approximately equal, or perhaps slightly larger, volume of air. The subcutaneous injections were made in the midline of the shoulder area. The injections of SF_6 or air were repeated at whatever intervals were necessary to maintain some inflation. The intervals between injections varied from one to two weeks. No precautions to maintain sterility were observed.

TABLE I

Treatment	Sex	No. of Survivors	Av. Body Weight (Gm.) Initial	Final
Air intrap.	M	8	149	412
SF_6 intrap.	M	9	151	400
Air subcut.	M	15	154	385
SF_6 subcut.	M	15	153	356
SF_6 subcut.	F	18	132	245
Air subcut.	F	13	129	244
SF_6 intrap.	F	14	128	242
Air intrap.	F	12	129	225

Body Weight.—Good weight gains were recorded by every group. In the case of the male rats, only those given subcutaneous injections of SF_6 showed a growth less than that typical of the colony norm (380 Gm. at the same age). A number of the rats had shown areas of infection in the inflated skin, which may account for the depressed growth. In the case of the female rats, only the group given air intraperitoneally grew somewhat less rapidly than the colony norm (235 Gm. in rats of the same age). The male rats given SF_6 subcutaneously showed the greatest growth retardation, whereas the female rats given SF_6 subcutaneously had the highest average weight. The deviations in the growth curves are probably coincidental; there is nothing in the growth curves to indicate a specific toxic effect of SF_6.

Mortality.—In the male rats given air or SF_6 subcutaneously, identical mortalities were observed throughout the experimental period. Negligible differences occurred when the two gases were administered intraperitoneally. The subcutaneous administration produced only 25% mortality; whereas given intraperitoneally, mortalities by the

end of the thirty-sixth week were 60 to 65%. In the female rats, higher mortalities were observed when air was administered than when SF_6 was administered. Given subcutaneously, the accumulated mortality with air injections reached about 35%; whereas with SF_6, the maximum was only 10% mortality. The total accumulated mortalities by the end of the period were 30 and 40%, respectively, when air and SF_6 were given intraperitoneally. No sterile precautions were used in injecting the gases. During the first month the rats did not respond adversely to the injections. After a period of about ten weeks, however, it became apparent that many rats were dying in the few days following each injection; fatal infections were suspected. Beginning at this time, penicillin was routinely administered parenterally simultaneously with the gas injections. Almost without exception, deaths no longer occurred in the days immediately following the gas injections. There is no indication in the mortality data that SF_6 is more toxic than air.

The higher mortality rates with intraperitoneal injections than with subcutaneous injections can be explained as an injection artifact. On several occasions it was observed that animals died immediately with peritoneal refills. Autopsy showed that in each case the needle had punctured a blood vessel and that large gas bubbles were present in the circulation. Such accidents never occurred when gas was given subcutaneously.

Organ Weights.—Rats were sacrificed after thirty-six weeks on the experimental regimen and gross autopsy examinations were conducted under the direction of Dr. Richard C. Crain of the Department of Pathology. A number of organs were dissected out and weighed, these included: the liver, kidney, lungs, brain, stomach, heart, and spleen. The average organ weights are given for each of the experimental groups in Table II. The average organ weights calculated on the basis of body weights are also presented for each group. All of the organ weights were within the normal ranges; there was no indication of any abnormality when SF_6 was injected, either subcutaneously or intraperitoneally.

Pathology.—Sections were prepared using hematoxylin and eosin stains of the following tissues: heart, lungs, spleen, stomach, small and large intestines, kidney, adrenal, testes or ovary, urinary bladder, bone marrow, and brain; in addition, any abnormal tissue was sampled for study. Sections of skin were removed from the shoulder area of the rats receiving subcutaneous administration of gas and sections of the abdominal wall were taken from those animals receiving gas intraperitoneally. The histological study was carried out by Dr. Robert D. Coye, Jr. of the Department of Pathology. Skin sections from three males and three female rats, given air or SF_6 intraperitoneally, and from the same number, injected subcutaneously, were also examined by Dr. D. L. Opdyke of the Procter and Gamble Company. These tissue samples were fixed in formalin solution. Routine paraffin sections were prepared and stained with hematoxylin and eosin.

No lesions were found that were attributed to a toxic effect of SF_6. Dr. Opdyke described normal blood vessels, collagen fibers, and sebaceous glands; hair follicles revealed no vesicles or any signs of chemical or structural changes (Fig. 1). There was no hyperplasia, keratization was normal, no evidence of infiltration, nor any indication of inflammatory reactions were seen. In a few rats, during the course of this study, areas of infection developed locally in the inflated skin above the subcutaneous injection. Presumably the pressure had reduced, at least in some measure, the circulation into the inflated areas of the skin. No lesions were found in the peritoneal tissue of the rats at the end of the study. The deaths observed during the experimental period were attributed to secondary infections. Of the 29 rats that died during the experiment, 14 were diagnosed at autopsy as dying of acute infection or of hemorrhage. These deaths were approximately equally divided between the control and the experimental groups. In many other cases, autopsy examinations were valueless because autolysis was advanced.

TABLE II.—AVERAGE ORGAN WEIGHTS

Sex	Treatment	Route	Liver	Kidneys	Testes	Lungs	Brain	Stomach	Heart	Spleen	No. Rats
M	Air	Subcut.	12.29	2.92	3.02	2.40	2.05	2.62	1.45	1.48	14[a]
M	Air	Intrap.	13.73	3.33	3.35	2.93	2.08	2.00	1.49	1.31	8
M	SF_6	Subcut.	11.62	2.86	3.08	2.20	1.90	1.89	1.45	1.47	15
M	SF_6	Intrap.	12.53	3.11	3.19	2.13	1.99	1.78	1.30	1.29	9
F	Air	Subcut.	7.83	1.95	...	1.66	1.86	1.51	1.03	1.09	13
F	Air	Intrap.	7.93	1.98	...	1.75	1.86	1.47	0.97	1.07	12
F	SF_6	Subcut.	8.42	2.07	...	1.85	1.89	1.56	0.99	0.95	17
F	SF_6	Intrap.	8.22	2.04	...	1.66	1.86	1.52	1.02	0.94	14
		Average Organ Weight/Body Weight Ratios (mg./Gm. b. wt.)									
M	Air	Subcut.	32.01	7.69	7.97	6.34	5.42	5.41	3.83	3.84	
M	Air	Intrap.	33.33	8.09	8.15	7.14	5.07	4.85	3.62	3.20	
M	SF_6	Subcut.	32.80	8.11	8.59	6.69	5.45	5.29	4.16	4.23	
M	SF_6	Intrap.	31.22	7.77	8.07	5.31	5.03	4.47	3.30	3.20	
F	Air	Subcut.	32.14	7.99	...	6.86	7.71	6.22	4.24	4.46	
F	Air	Intrap.	35.17	8.82	...	7.83	8.28	6.55	4.34	4.70	
F	SF_6	Subcut.	34.40	8.48	...	7.60	7.76	6.42	4.05	3.89	
F	SF_6	Intrap.	33.88	8.40	...	6.82	7.69	6.25	4.19	3.86	

[a] Liver weights on 15 rats; brain weights on 13 rats.

Fig. 1.—Section through the skin area adjacent to the gas space. The gas space is lined by a uniform organized connective tissue wall which shows acidophilic staining connective tissue with numerous capillaries and which, with Mallory stains, reacts positively for collagen.

From a thorough histological study, the surviving rats were found to exhibit the lesions commonly found in rats of this age in our colony. Low grade chronic pulmonary infection and low grade kidney infection were often seen. A number of parasites were observed in the liver and the urinary bladder. Only one tumor was found—a sarcoma of undetermined origin. One unusual lesion was described as a hamartoma of loose connective tissue with prominent capillaries and a few irregularly arranged epithelial cells forming duct-like structures.

A special search was made for any effects that might be attributed to a contamination with trace amounts of S_2F_{10}. This compound is known to produce pulmonary edema, and questionable damage to the epithelium of large bronchi and to the trachea when the gas is inhaled (11). None of these changes could be found in any of the rats examined.

In summary, groups of 20 male and 20 female, young adult, albino rats were treated and observed over a period of six months. Each experimental rat was injected with a volume of SF_6, either intraperitoneally or subcutaneously. Each control rat was injected with a similar volume of air. The injections were repeated for both groups simultaneously. Infections were controlled with injections of penicillin to obviate deaths following the treatment days. Mortalities were comparable for the control and experimental rats. The organ weights of rats surviving the six-months' period lay in normal ranges. A thorough histological study revealed no evidence of any tissue lesion that was attributed to the treatment. SF_6 was neither toxic nor irritating under the conditions of this study.

DOG STUDIES

Two dogs were maintained with pneumoperitoneum refills for periods of six months or longer. A third dog that died after the second injection of SF_6 showed on autopsy evidence of what is taken to be an intercurrent illness, *viz.*, a focal bronchial pneumonia, a generalized pulmonary congestion, hemoperitoneum, and hemopericardium.

The two dogs that survived the six months' experimental period were treated on the schedule shown in Table III. Gas was introduced into the peritoneal cavity by a 100-cc. syringe and a semiblunt needle equipped with a simple rubber bag containing the gas. Injections were always made at approximately the same site in the lower quarter of the abdomen. Before introducing the SF_6, the residual gas within the peritoneum was removed. These two dogs were normal, young, well-nourished male beagles. The dogs had good appetites during the test period. During a period of several months, one dog lost weight, *viz.*, the body weight fell from 11.8 to 9.7 Kg.; some of this weight was regained before the end of the experimental period.

TABLE III.—TREATMENT PROGRAM FOR DOGS; BODY WEIGHTS

	Dates of SF_6 Refills	Body Wt. (Kg.)
Dog No. 2999 M	5–12–54	14.8
	6–9–54	15.2
	7–14–54	15.9
	7–17–54 Dead	
Dog No. 2081 M		
Initial	4–14–54	11.8
3 mo.	7–14–54	10.9
6 mo.	10–14–54	10.4
9 mo.	1–12–55	9.7
Termination	3–3–55	10.0
Dog No. 2823 M		
Initial	8–11–54	6.3
3 mo.	11–10–54	6.6
6 mo.	2–9–55	6.1
Termination	3–3–55	6.3

Blood counts were obtained for each dog toward the end of the experimental period. The hematological values recorded included red blood cell counts, hemoglobin values, white blood cell counts, differential counts, and observations of a number of characteristics of the red blood cells. All values were in the normal ranges.

Urine samples were collected from one of the dogs (No. 2823) and examined by the usual semiquantitative methods for sugar and protein—only normal trace amounts were present.

Organ Weights.—The dogs were sacrificed by the intravenous administration of a large dose of a soluble barbiturate. A number of organs were dissected out and weighed (Table IV). The organ weights were within the normal ranges.

Pathology.—When the dogs were sacrificed, gross autopsy examinations were made by Dr. R. D. Neubecker of the Department of Pathology. Sections of the following tissues were made and stained with the customary hematoxylin and eosin

TABLE IV.—ORGAN WEIGHTS OF DOGS

	Organ Wt. (fresh)		Organ Wt. Basis Body Wt. (Gm./kg.)	
	No. 2081	No. 2823	No. 2081	No. 2823
Heart	90	46	9.0	7.3
Lungs	R 53	38	5.3	6.0
	L 40	30	4.0	4.8
Spleen	43	28	4.3	4.4
Liver	314	234	31.4	37.1
Kidneys	R 41	24	4.1	3.8
	L 41	24	4.1	3.8
Brain	78	67	7.8	10.6

stains: heart, lung, spleen, pancreas, stomach, small and large intestine, liver, kidney, urinary bladder, lymph nodes, omentum, peritoneum, diaphragm, abdominal wall, adrenal, testes, thyroid, bone marrow, and brain.

Neither dog showed any gross or histological changes that were attributed to the SF_6 treatment. One dog (No. 2081) showed a mild pyelitis and had focal granulomata in the lung and lymph nodes. The pleural cavities were normal. No excess fluid was present in the peritoneal space. The peritoneal surfaces were thin and normal. Specifically there was no inflammation or scarring in the omentum, or the diaphragm, or in the tissues most closely exposed in the peritoneal cavity and in the pleural cavity. One dog had some cystitis, a mild pyelitis, and a vesicle calculus. Lung sections were normal with the exception of some scattered intra-alveolar hemorrhage.

Both of these dogs were normal, well-developed young dogs with no histological evidence of any effect of SF_6. As with the rats, a careful search revealed no indication that a material having the properties of S_2F_{10} had been present in any detectable trace.

DISCUSSION

Based on the evidence of the lack of toxicity in the animal studies, two clinical trials were conducted (10): one series on three volunteer patients at the Iola Sanitarium in Rochester, N. Y.; the other on ten patients undergoing treatment for tuberculosis in the Veterans' Hospital, Batavia, N. Y.

In Table V, the records of treatment periods, volumes of gases, and frequencies of refillings are presented. The patients were examined fluoroscopically to observe the volume changes of the gas pockets and the gas pressures were measured directly before and after each refill. No untoward symptoms or physical signs were recorded during the course of SF_6 pneumoperitoneum. The longer periods between refills, *viz.*, five or six weeks with SF_6 *vs.* ten to fourteen days with air, and the lessened discomfort because needle injections were less frequent were greatly appreciated by the patients.

The patients in the Veterans' Hospital group received SF_6 injections over periods of three to nine months. Refill volumes of 800 to 1,000 cc. were required at intervals of five to seven weeks, in contrast to air refill volumes 100 to 200 cc. larger at intervals of ten to fourteen days. Records of temperature, pulse, hemoglobin, white blood cell count, bromsulphalein retention, blood urea nitrogen, and phenosulfonphthalein tests were negative, i. e., functions were unimpaired.

As a result of the preliminary trials, SF_6 is recommended (10) as "an ideal gas for use in therapeutic artificial pneumoperitoneum and should also be tried clinically in artificial pneumothorax."

SUMMARY

1. Groups of 20 male and 20 female rats, each, were treated (*a*) with SF_6 subcutaneously, (*b*) with SF_6 intraperitoneally, (*c*) with air subcutaneously, and (*d*) with air intraperitoneally, for a period of approximately six months. The intervals between injections varied from one to two weeks. Each group grew normally.

2. Deaths, presumably from infection, occurred during the early part of the experiment; the administration of penicillin simultaneously with the air or SF_6 injections controlled the mortalities. Mortality rates were no greater when SF_6 was administered than when air was administered.

3. Organ weights lay in the normal ranges.

4. In a thorough histological study, no lesions were found that were attributed to the treatment by SF_6. Specifically, the skin and membranes that had been in contact with SF_6 or with air showed no evidence of toxicity or irritation.

5. Two dogs were maintained with pneumoperitoneum of SF_6, one dog for ten months and the other dog for nearly seven months. Gas was administered at intervals varying from one week

TABLE V

	Air	SF_6
Iola Case No. 1	March 1951–Sept. 1952 1,200 cc. every 10 days	Sept. 1952–April 1954 1,000 cc. every 6 weeks.
Iola Case No. 2	July 1951–Sept. 1952 1,000 cc. every 10 days April 1954– 1,000 cc. every 2 weeks	Sept. 1952–April 1954 800–900 cc. every 5 weeks.
Iola Case No. 3	April 1954 1,400 cc. every two weeks	Oct. 1953–April 1954 1,200 cc. every 5 weeks.

to three weeks. Body weights were maintained by one dog, the other dog lost some weight but regained most of it by the end of the period. Organ weights lay in the normal ranges. A thorough histological study revealed no tissue change that was attributed to the administration of SF_6.

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A Study of the *In Vitro* Reaction of Catalase and Hemin with Organic Mercury Compounds*

By SISTER M. ANGELICE SEIBERT

The relative inhibitory effect of a series of organic mercury compounds (phenylmercuric nitrate, phenylmercuric hydroxide, *p*-chloromercuribenzoic acid, Neohydrin, and Mercuhydrin) on the catalytic activity of catalase *in vitro* was studied. The inhibition is relatively less with the diuretics. Increasing the concentration of all of the mercury compounds gives greater inhibition. Spectrophotometric studies of catalase protein, Armour serum albumin and crystalline hemin were made. Interaction of the protein —SH with the organic mercury compounds does not explain the inhibition under the conditions of this study. The catalase activity of hemin is inhibited by the mercury compounds in a system completely devoid of —SH groups. The hemin itself is rapidly destroyed by the hydrogen peroxide and this reaction is also depressed by the organic mercury compounds. There is no spectroscopic evidence for a hemin-mercury or hydrogen peroxide-mercury compound. Spectroscopic and preliminary polarographic data indicate the necessity of postulating a ternary complex, hemin-hydrogen peroxide-organic mercury, which is relatively stable.

AT PRESENT there is a variety and unfortunate confusion of concepts relating to the mode of action *in vitro* and *in vivo* of several types of mercury compounds falling under the classification of organic mercury compounds.

p-Chloromercuribenzoic acid (PCMB) has been considered a selective reagent for —SH binding and its reversible inhibition of enzyme activity has led to the expression "sulfhydryl-dependent enzymes" (1). However, the simple inhibition of enzyme activity by PCMB does not demonstrate conclusively that —SH groups are essential for the activity of a given enzyme (2, 3).

Phenylmercuric nitrate (PMN) and phenylmercuric hydroxide (PMOH) have been shown to inhibit *in vitro* in a non-reversible manner enzymes supposedly not requiring —SH groups for activity (4). The *in vitro* studies of Goldstein and Doherty (5) with cholinesterase indicate that the problem of the influence of mercury compounds on enzyme activity is not adequately explained in some instances in terms of —SH binding. The demonstration of a direct interaction of organic mercury compounds with thyroxine and related compounds under mild conditions by Frieden and Naile (6) is also of interest to this problem.

Organic mercury compounds of the diuretic group inhibit both —SH and non—SH dependent enzymes (7). Ruskin and Ruskin (8) have suggested that the action of Mercuhydrin may be at two different sites of the terminal oxidative chain. Riggs and Wolbach (9) investigating the inhibitory effects of organic mercury compounds on the autocatalytic binding of hemoglobin with oxygen conclude that the mercury compounds inhibit the heme-heme interaction by blocking or modi-

* Received July 5, 1957 from the Research Laboratory, Ursuline College, Louisville 6, Ky., an affiliated laboratory of the Institutum Divi Thomae Foundation, Cincinnati, Ohio.
This study was supported in part by funds from Research Corporation, N. Y. and the Damon Runyon Memorial Fund.
The author wishes to thank Dr. C. W. Kreke, Mount Mercy College, Pittsburgh, for his valuable criticism of this work; and Miss Kathleen Pfaum for technical assistance.

fying structural reorganization which accompanies the oxygenation.

Earlier work (4, 10, 11) has shown that catalase is inhibited by PCMB, PMN and PMOH. This inhibition is relatively mild as compared with that observed with cytochrome oxidase and succinic dehydrogenase. Since this inhibition of catalase was apparently not a simple binding of essential —SH groups, it was of interest to investigate further the mode of action of these mercury compounds and to compare the effects of these compounds with those of several organic mercury diuretics.

EXPERIMENTAL

All spectrophotometric measurements were made with a Beckman Model DU spectrophotometer employing a water cooled lamphouse and cell compartment.

Polarographic measurements were made with a Sargent Model XII Camera Recording Polarograph. An H cell with a saturated calomel electrode and a KCl agar bridge was used as the anode. Oxygen was removed from the test solutions by passage of oxygen-free nitrogen. All measurements were carried out at 25 ± 0.1°. The capillary had the following characteristics: t = 3.3 sec. at a pressure of 48 cm. of mercury on an open circuit in the test solution. The weight of the mercury dropping per second, m, was 2.04 mg. All solutions were buffered to a pH of 7.4 with 0.1 *M* phosphate buffer and were examined over a potential range of 0.0 to −1.8 v.

Armour No. 30 pork liver catalase[1] was used for all the enzyme experiments. Crystalline hemin was purchased from Nutritional Biochemicals.

The stock solution of catalase was prepared by dissolving a weighed amount of the powdered enzyme in 0.1 *M* phosphate buffer, pH 7.4, and diluting to the required concentration for use. The final concentration of the enzyme was determined spectrophotometrically at 406 mμ as described in a previous paper (4).

The hemin crystals were dissolved in a few drops of 6 *N* NaOH and several ml. of glass-distilled water. The solution was then made up to volume with buffer. The concentration was calculated from an accurately weighed sample of crystals using a molecular weight of 675.55. The stock solution was 4.4 × 10^{-3} *M*.

Basic phenylmercuric nitrate and phenylmercuric hydroxide were purchased from Hamilton Laboratories, Inc.; PCMB from the Schaeffer Laboratories, Inc. Mercuhydrin and Neohydrin were obtained from Lakeside Laboratories, Inc., Wisconsin.[1]

Catalase and hemin activity was determined by following the breakdown of hydrogen peroxide at 240 mμ according to the tracking method of Beers and Sizer (12) with some modifications.

In all the experiments the catalase or hemin and the inhibitor were premixed and allowed to stand for one hour before adding the substrate. The catalase solutions were kept at 10° at all times; the hemin solutions at 25°.

An approximately 4.5 × 10^{-3} *M* H_2O_2 solution was prepared by diluting 1.2 ml. of Mallinckrodt, A.R., 3% H_2O_2 with 25 ml. of 0.1 *M* phosphate buffer pH 7.4, for the catalase experiments. For the hemin experiments a 8.8 × 10^{-2} *M* solution was used.

In the catalase experiments 2 ml. of the enzyme solution were placed in both the blank and experimental cell. One ml. of buffer was added to the blank. At zero time, 1.0 ml. of the H_2O_2 was added to the enzyme solution and the absorbance at 240 mμ read every ten seconds for a ninety second period.

In the hemin experiments, 3.0 ml. of the hemin solution was added to the experimental cell. The blank contained 3.0 ml. of either buffer or of the buffer and mercury compound. At zero time, 0.5 ml. of H_2O_2 was added to the experimental cell and the change in absorbance was followed with time. Final concentration of the hemin in the spectrophotometer cell was 2.7 × 10^{-6} *M*.

RESULTS AND DISCUSSION

Catalase studies: Table I shows the comparative effect of five organic mercury compounds on the activity of Armour catalase No. 30. The per cent depression is calculated from the rates of change of the absorbance during a one minute interval (from twenty to eighty seconds) of a control catalase solution and one containing the stated molar concentration of mercury compound. Concentration of the mercury compounds are final molar concentration in the reaction mixture.

TABLE I.—DEPRESSION OF CATALASE ACTIVITY BY ORGANIC MERCURY COMPOUNDS

Hg Compd. (1.4 × 10^{-4} *M*)	% Depression
Phenylmercuric nitrate	23
p-Chloromercuribenzoic acid	16[a]
Phenylmercuric hydroxide	8
Neohydrin	5
Mercuhydrin	0

[a] This depression was obtained with a concentration of 1.4 × 10^{-5} *M*. Higher concentrations were too absorbing to permit spectrophotometric measurements.

The concentration of catalase in each of the reaction mixtures was 1.12 × 10^{-8} *M*. Increasing the concentration of the mercury compounds gave increased inhibition. Mercuhydrin could not be used in concentrations greater than 5 × 10^{-4} *M* because of high absorbance. The effect of increasing amounts of Neohydrin is shown in Fig. 1.

Activity experiments measuring the effect of the mercury compounds on the ability of hemin to breakdown H_2O_2 showed significant inhibition of this property of hemin. Table II summarizes these data. The final molar concentration of the mercury compounds was 1.4 × 10^{-4} *M*; hemin, 2.7 × 10^{-6} *M*; H_2O_2, 1.25 × 10^{-2} *M*. The temperature was 25°.

The effect of varying the concentration of Neohydrin and PMN is shown in Fig. 2.

A study of possible mercaptide formation between the mercury atom of the organic mercury compounds

[1] These materials were generously supplied without charge.

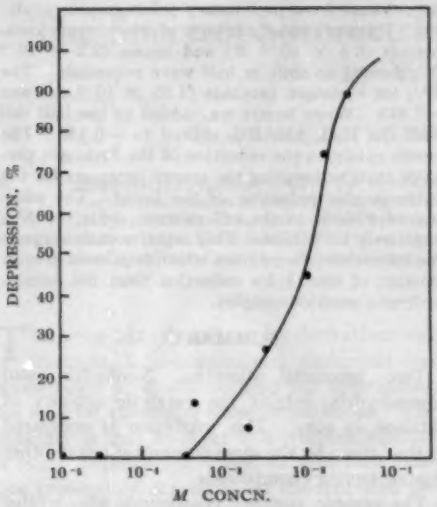

Fig. 1.—Effect of Neohydrin on the activity of Armour Catalase No. 30.

TABLE II.—DEPRESSION OF THE CATALYTIC ACTIVITY OF HEMIN BY ORGANIC HG COMPOUNDS

Hg Compds.	Depression, %
Phenylmercuric nitrate	75
Neohydrin	50
Phenylmercuric hydroxide	37
Mercuhydrin	14

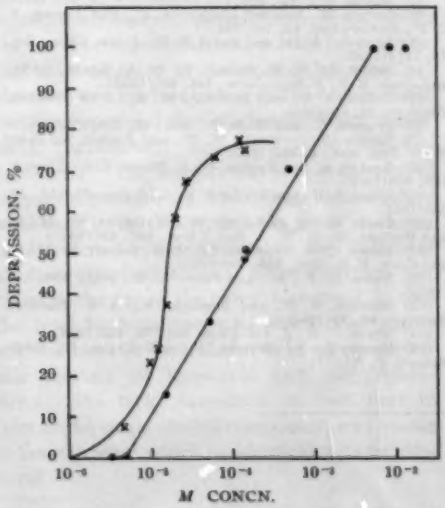

Fig. 2.—Effect of organic mercury compounds on the catalase activity of crystalline hemin. ● Neohydrin; X Phenylmercuric nitrate.

and the —SH groups of catalase protein and Armour bovine serum albumin[1] indicated that under the conditions of these experiments very little mercaptide formation occurs as judged by increase of absorbance of the solutions at 250 mμ. Measurements were made according to the procedure used by Boyer (14). In any case the concentration of mercury compound employed in the catalase activity experiments is in considerable excess of that required for —SH binding. For these studies Armour catalase No. 30 was used without further purification. This preparation contains only 24% catalase protein. Therefore, in order to prepare a solution of catalase protein an amount 24% greater than the calculated amount had to be dissolved. This resulted in a protein solution having a very high nonspecific protein absorbance. The very small increase in absorbance obtained in mixtures of this solution and the mercury compounds could be attributed almost completely to a non-specific reaction of the mercury compounds with the non-catalase protein. This interpretation is substantiated by earlier work (4) which showed that a very pure crystalline catalase preparation when mixed with PMN or PMOH showed no spectral shifts nor absorbance increase over the entire spectral range from 230 mμ to 700 mμ. Part of the action of the mercury compounds must then involve the hematin portion of the catalase molecule.

It was reported in an earlier publication that the hemin spectrum is not affected by relatively high concentrations of PMN and PMOH and PCMB (4). This is in agreement with the observations of Riggs and Wolbach (9) that neither mercuric chloride, Mersalyl, PCMB nor methyl mercury hydroxide alter the spectrum of free ferriheme. Benesch and Benesch (13) also report that PMOH and PCMB have no effect on the absorption spectrum of hemoglobin.

Measurements of the absorption spectra of mixtures of the mercury diuretics, Neohydrin and Mercuhydrin, with hemin between 230 mμ and 650 mμ showed no alteration of the hemin or mercury compound spectra. Likewise no spectral changes were demonstrated in mixtures of the mercury compounds with hydrogen peroxide. Mixtures of hemin and hydrogen peroxide and of hemin, hydrogen peroxide and PMN in concentrations used in the activity experiments showed no differences in absorption spectra with the exception of a drop in absorbance due to the decomposition of the hydrogen peroxide and of the hemin. The spectra were run within five minutes after adding the hydrogen peroxide. Haurowitz, *et al.* (15), report a short-lived addition product of ferri-heme with hydrogen peroxide in aqueous media. This product is stabilized in pyridine and gives a characteristic absorption spectrum.

Under the conditions of our experiments, hemin is rapidly destroyed by the hydrogen peroxide during the reaction as shown by spectral evidence. The rate of destruction of hemin was measured by the same method as was used in following the breakdown of hydrogen peroxide except that the absorbance was read at the 300 mμ and 580 mμ maxima of hemin. The change in absorbance during the thirty minute period from five to thirty-five minutes is shown in Table III. The decomposition of hemin

is considerably faster at 25° than at 10° and the PMN inhibition of the decomposition reaction is relatively greater at the higher temperature.

TABLE III. EFFECT OF PMN ON THE DESTRUCTION OF HEMIN BY HYDROGEN PEROXIDE

	Δ In Absorbance 10° at 390 mμ	25° at 390 mμ	25° at 580 mμ
Hemin	0.308	0.638	0.075
Hemin-PMN	0.233	0.380	0.040

The reaction of hemin and hydrogen peroxide would appear to be as follows:

$$\text{Hemin} + H_2O_2 \underset{a}{\overset{b}{\rightleftharpoons}} \text{Hemin-}H_2O_2 \text{ (complex)}$$

$$\text{Hemin-}H_2O_2 \rightarrow \text{Products of } H_2O_2 \text{ decomposition} \quad \text{(I)}$$

$$\text{Hemin-}H_2O_2 \rightarrow \text{Decomposition of Hemin} \quad \text{(II)}$$

Normally in the case of catalase reaction (I) is faster than reaction (II). Our data with hemin show that reaction (II) is the faster under the conditions stated. The organic mercuric compounds inhibit both reactions (I) and (II). It does not seem necessary nor justifiable to postulate two different modes of action for the mercury compounds. In both instances they apparently work by preventing the breakdown of the hemin-H_2O_2 complex. Since we have no evidence for a hemin-mercury or a hydrogen peroxide-mercury complex, the mercury compounds apparently do not inhibit reaction (*a*). Inhibition of (*b*) would result in the accumulation of the hemin-hydrogen peroxide complex making it observable spectroscopically. For this we have no evidence. The data, then, suggest a ternary compound which does not decompose as rapidly as reaction (I) or (II) thus inhibiting both the catalytic property of the free hemin molecule and the decomposition of hemin by hydrogen peroxide.

$$\text{Hemin} + H_2O_2 + \text{Hg compd.} \underset{a}{\overset{b}{\rightleftharpoons}}$$

$$\text{Hemin-}H_2O_2\text{-Hg compd} \rightarrow \text{Products of } H_2O_2 \text{ decomposition} \quad \text{(III)}$$

$$\text{Hemin-}H_2O_2\text{-Hg compd} \rightarrow \text{Decomposition of Hemin} \quad \text{(IV)}$$

The rate of hydrogen peroxide breakdown by catalase is constant both in the control and mercury treated solutions. The rate of the hemin reactions (I) to (IV) decreases with time due to the simultaneous decomposition of the hydrogen peroxide and the hemin.

This hypothesis of a ternary complex is supported by evidence from preliminary polarographic analyses. Polarograms of mixtures of the mercury compounds (6.5×10^{-4} *M*) and hemin (2.2×10^{-5} *M*) showed no shift in half wave potentials. The $E^1/_2$ for hydrogen peroxide (1.25×10^{-3} *M*) was −0.849. When hemin was added to the half cell with the H_2O_2, the $E^1/_2$ shifted to −0.458. The hemin catalyzes the reduction of the hydrogen peroxide thereby lowering the energy necessary for the polarographic reduction of the latter. The addition of PMOH to the cell mixture shifts the $E^1/_2$ negatively to −0.714. This negative shift suggests the formation of a complex which requires a greater amount of energy for reduction than the hemin-hydrogen peroxide complex.

SUMMARY

Two mercurial diuretics, Neohydrin and Mercuhydrin, inhibit the catalytic activity of catalase *in vitro*. This inhibition is compared to that given by the same concentrations of other organic mercury compounds.

The organic mercury compounds also inhibit the catalase activity of purified hemin in a system completely devoid of —SH groups. The destruction of hemin by hydrogen peroxide is also inhibited by these compounds.

Spectroscopic and polarographic data indicate the necessity of postulating a hemin-hydrogen peroxide-organic mercury complex which is relatively stable.

The inhibition of the enzymatic activity of catalase is suggested by analogy to involve the formation of a catalase porphyrin-hydrogen peroxide-organic mercury complex.

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The Spectrophotometric Determination of Sterols in Wool Fat*

By BLAKE F. PUTNEY and ROBERT CALVO

Irregularities encountered in the application of the Liebermann-Burchard reaction to the determination of cholesterol, isocholesterol and their esters in wool fat unsaponifiables and wool fat have been investigated. Times and temperatures of color formation have been found critical in the determination of absorptivities and their analytical application. A satisfactory analytical procedure has been developed.

THE WIDE USE of wool fat, its derivatives and fractions, in cosmetics and medicinals has necessitated a reliable method for determining the wool fat content of these preparations. The difficulty is that wool fat is a complex mixture of many constituents, which vary in their relative proportions depending on the source of the wool and also on the process of extraction and purification. The monograph of wool fat U. S. P. is scarcely more than a simple physical description of the product and gives little indication of its chemical composition.

The commercial availability of several wool fat fractions suggested to the authors that various studies be carried out on these fractions to gain some insight into their chemical composition. The spectrophotometric determination of cholesterol and isocholesterol appeared to be a good starting point for a chemical investigation of these products.

The chemistry of wool fat has been reviewed by Lederer and Velluz (1) and Truter (2). The name, isocholesterol, as originally proposed for the steroid component found in wool fat unsaponifiable (in addition to cholesterol) has now been shown to be a mixture of four distinct compounds; namely, lanosterol (lanostadienol), dihydrolanosterol (lanostenol), agnosterol, and dihydroagnosterol. The mixture has also been referred to quite commonly as "the triterpene alcohols of wool wax." In this report, the name isocholesterol, refers to the mixture of the triterpene alcohols described above. Some confusion also exists in the use of the name, lanosterol, in that the wool fat component to which it originally referred has now been shown to be an equimolecular mixture of lanosterol and dihydrolanosterol. The term, lanosterol, is used here to refer to the single alcohol component, also known as lanostadienol which us identical with kryptosterol.

* Received May 7, 1957, from the laboratories of Rutgers University College of Pharmacy, Newark 4, New Jersey.
Presented to the Scientific Section, A.PH.A., New York meeting, May 2, 1957.

The Liebermann-Burchard reaction for quantitative determination of cholesterol was first proposed by Grigaut in 1910, and has since been used extensively with many modifications (3) to determine the cholesterol content of the blood and other body fluids. Other spectrophotometric methods for determinations of cholesterol in blood have been reported by Pearson (4) and Zlatkis (5); no report was found of the use of the latter methods in determination of cholesterol or isocholesterol in wool fat. A gravimetric method for cholesterol in blood has also been widely used, but its use in wool fat analysis has been seriously questioned by Knol (6).

The different colors produced by cholesterol and isocholesterol when treated with L.-B. reagent were first observed by Schulze in 1890, and studied extensively by Lederer and Tchen (7, 8). The latter observed that, essentially, the same color reaction was given by the other sterols of the isocholesterol fraction as given by lanosterol, and suggested the use of lanosterol as a standard for the determination of the isocholesterol content of wool fat.

The application of the L.-B. reagent to cholesterol esters has shown that the color is produced more rapidly and that the absorbance is greater by approximately 20 per cent for equivalent cholesterol content than in the case of free cholesterol (9). Similarly, Lederer and Tchen (7) reported a difference of about 5 per cent in the color produced by lanosterol and lanosteryl acetate.

A recent publication by Luddy, *et al.* (10), reported extensive work on the determination of cholesterol and isocholesterol (triterpene alcohols) in wool fat. The chief modifications in this procedure involved the determination of isocholesterol at 550 mμ, instead of the wavelength of the primary maximum at 450 mμ (Fig. 1); use of a spectrophotometer; and development of the color complexes at room temperature. This procedure was questioned because the temperature was not controlled and standard solutions

of cholesterol and lanosterol were not run under the same conditions of time and temperature as the unknown solutions. Duewell (11) has also reported use of the L.-B. reaction for determination of cholesterol and isocholesterol in wool fat.

EXPERIMENTAL

Equipment.—A Bausch and Lomb Spectronic-20 Spectrophotometer, equipped with a Thomas Roto-Cell Carrier Assembly[1] with a partitioned Corex cell having a light path of 1 cm., was used. The carrier was equipped with a liquid cooling chamber through which water was passed in order to maintain the desired temperature.

The samples were prepared in glass-stoppered 25-cc. mixing cylinders of low actinic glass.

Reagents.—The cholesterol (Fisher Scientific Co.) was recrystallized from alcohol-water and dried at 80° for three hours, m. p.[2] 148.5–9.0°; $[\alpha]_{25}^{D}$ ($CHCl_3$), −38.8°.

Cholesteryl acetate (Brothers Chemical Co.) was recrystallized from acetone and dried at 80° for three hours, m.p. 113.5–114.0°.

Initially, the lanosterol used was obtained by isolation from wool fat by the method of Doree and Garratt (12), m. p. 138–138.5°. Later, a sample of "pure lanosterol" (Organon Laboratories Ltd., London, England)[3] m. p. 137.5–138.0°, $[\alpha]_{25}^{D}$ ($CHCl_3$), +56.5°, was used.

Lanosteryl acetate was prepared by refluxing lanosterol with an excess of acetic anhydride, crystallized from alcohol or acetone and dried at 80° for three hours, m. p. 123–125°. The acetate prepared from the commercial lanosterol, after drying at 80° for three hours, had a m. p. of 126–128°.

Special peroxide-free 1,4-dioxane (Fisher Scientific Co., cat. no. D-111) was used.

Reagent grade chloroform, sulfuric acid, acetic anhydride, acetic acid, and wool fat, U.S.P., were used.

The Liebermann-Burchard reagent was prepared by the method of Luddy (10): 1 volume of cold (0°) concentrated sulfuric acid was added to 4 volumes of acetic anhydride immersed in an ice-salt bath. The acid was added slowly with shaking to prevent discoloration of the reagent at this point. The acid-anhydride mixture was then diluted with 2.5 volumes each of glacial acetic acid and 1,4-dioxane and stored at 0°. The reagent is relatively unstable and should only be used on the day of its preparation. It should be rejected if a brown color develops during use.

Stock solutions.—Stock solutions of the sterols and their acetates were prepared in reagent grade chloroform. The solutions had concentrations in the range of 6 to 12 mg. of sterol in 100 cc. of solution. Wool fat solutions were prepared to contain 25 to 35 mg. of wool fat in 100 cc. of solution. The solutions of wool fat unsaponifiable were made to contain 12 to 16 mg. of unsaponifiable in 100 cc. of solution.

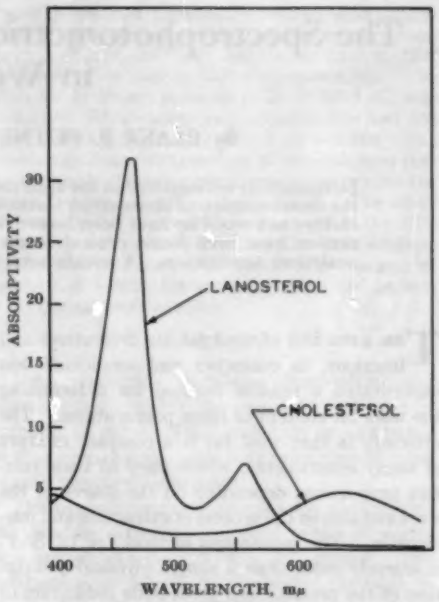

Fig. 1.—Plot showing the wavelength of maximum color absorption of the Liebermann-Burchard color complexes of cholesterol and lanosterol (10).

Procedure.—Ten cubic centimeters of the solution to be analyzed was pipetted into a 25-cc. mixing cylinder and 5 cc. of cold L.-B. reagent was added. A blank consisting of 10 cc. of chloroform and 5 cc. of L.-B. reagent was prepared simultaneously. Immediately after adding the reagent, a stop watch was started, the cylinder inverted several times and placed in a constant temperature bath, maintained at 30° and protected from light. The length of time for a sample in the bath varied, as indicated in Table I, in order to allow the color to reach its maximum intensity after the solution was placed in the spectrophotometer. Water from the bath, at 30°, was passed through the cooling chamber of the carrier assembly.

Absorbances were taken at two-minute intervals, reading first at 550 mμ and then at 630 mμ. During the assay, the cell was turned so that light passed through the solution *only while readings were being taken.*

Calculations.—In determining the absorptivities, the absorbances of cholesterol, lanosterol, and their respective acetates were taken at 630 mμ (the wavelength at which maximum light absorption occurs for cholesterol and its esters) and at 550 mμ (the wavelength at which a secondary maximum of absorption occurs for lanosterol and its esters) Fig. 1. This was done in order to take into account the colored isocholesterol derivatives having appreciable absorption at the wavelength

[1] A. H. Thomas Co., Phila., Pa.
[2] All m.p.'s are corrected.
[3] The authors wish to express their appreciation to Mr. E. A. Newcomb of the Malmstrom Chemical Corp., Newark, N. J., for obtaining this sample.

at which the cholesterol color complex has its maximum and vice versa. From these data the absorbtivity was calculated as follows:

Absorptivity (a_s) = Absorbance/Concn. (Gm./L.) × length of light path (cm.)

These absorptivities (Table II) were then used in the calculation of cholesterol-lanosterol mixtures (Table III) and cholesteryl acetate-lanosteryl acetate mixtures (Table IV). The following equations were employed to determine the amounts of the respective sterols in the solutions: Cholesterol (Gm./L.) = $(A_{630}a_L - A_{550}a_L')/(a_La_C - a_L'a_C')$; Lanosterol (Gm./L.) = $(A_{550}a_C - A_{630}a_C')/(a_La_C - a_L'a_C')$ in which A_{630} and A_{550} are the absorbances of the solution at the respective wavelengths; a_C and a_C' are the absorptivities of cholesterol (or cholesteryl acetate) at 630 and 550 mμ, respectively; a_L and a_L' are the absorptivities of lanosterol (or lanosteryl acetate) at 550 and 630 mμ respectively.

In order to determing the significance of the rate of color development on the results obtained in the following analyses, the calculations were carried out in two ways.

Method I. —The absorbances employed in the calculations were those observed at the time of maximum color development for the sterols being determined. For example, on calculation of a mixture of cholesteryl acetate and lanosteryl acetate, the absorbances were those observed at 18 minutes at 630 mμ and 30 minutes at 550 mμ. The absorptivities employed were those previously found at the corresponding times (Table II), a_C and a_L' at 18 minutes and a_L and a_C' at 30 minutes. In the analysis of cholesterol-lanosterol mixtures, absorbances

TABLE I.—TIME ALLOWED FOR COLOR DEVELOPMENT AND READING OF ABSORBANCE OF SOLUTIONS

Sample	Time in bath, Min.	Reading Times, Min. Init.	Final	Maximum Color Time, Min.	Wavelength, mμ
Cholesterol	18	20	32	24	630
Cholesteryl Acetate	10	14	32	18	630
Lanosterol	20	24	32	28	550
Lanosteryl Acetate	15	18	34	30	550
Wool Fat or Acetate mixtures	10	14	32	18	630
				30	550
Wool fat unsaponifiables or free sterol mixtures	18	20	32	24	630
				28	550

TABLE II.—ABSORPTIVITIES OF LIEBERMANN-BURCHARD COLOR COMPLEXES OF STEROL COMPONENTS OF WOOL FAT

Time, (Min.)	Cholesterol 630 mμ	Cholesterol 550 mμ	Chol. Acetate 630 mμ	Chol. Acetate 550 mμ	Lanosterol 630 mμ	Lanosterol 550 mμ	Lano. Acetate 630 mμ	Lano. Acetate 550 mμ
14	..	..	5.35	1.77	..	..	..	..
16	..	..	5.44	1.76	..	..	0.28	4.00
18	4.75	1.59	5.43	1.71	..	..	0.30	4.51
20	4.84	1.60	5.34	1.65	..	..	0.32	5.05
22	4.88	1.60	5.26	1.60	..	..	0.32	5.41
24	4.92	1.59	5.13	1.60	0.47	5.19	0.32	5.52
26	4.86	1.58	5.02	1.54	0.49	5.33	0.36	5.72
28	4.79	1.54	4.88	1.56	0.51	5.44	0.34	5.89
30	4.72	1.53	4.76	1.52	0.53	5.44	0.34	5.95
32	4.63	..	4.64	..	0.54	5.37	0.34	5.90
34	..	..	..	..	0.56	5.25	0.34	5.89

TABLE III.—RESULTS OF ANALYSIS OF CHOLESTEROL-LANOSTEROL MIXTURES AND OF WOOL FAT UNSAPONIFIABLES AND COMPARISON OF RESULTS OBTAINED BY TWO METHODS OF CALCULATION

Sample	Cholesterol Taken[a]	Found[b], % Method I[c]	Method II[c]	Lanosterol Taken[a]	Found[b], % Method I[c]	Method II[c]
147-A[d]	0.0243	100.3	100.3	0.0209	102.4	102.7
147-B[d]	0.0318	103.1	104.0	0.0390	100.6	100.4
147-C[d]	0.0492	102.4	102.6	0.0309	103.1	103.3
147-D[d]	0.0299	101.7	97.7	0.0390	100.3	103.0
Average		101.9	101.0	..	101.6	102.4
152-A[e]	0.1108	41.7	40.9	..	28.2	28.8
152-B[e]	0.1089	41.5	41.5	..	28.1	28.7
Average		41.6	41.2	..	28.2	28.8

[a] The concentration of sterol (or wool fat unsaponifiable) in the color solution expressed as Gm./L.
[b] Percentage of known amount of component, found on analysis of mixtures of sterols. For wool fat unsaponifiables, percentage of cholesterol or isocholesterol (calculated as lanosterol) in wool fat unsaponifiable.
[c] See text. [d] Known mixtures of sterols. [e] Wool fat unsaponifiable.

were those observed at 24 minutes at 630 mμ and 28 minutes at 550 mμ; absorptivities were also at 24 and 28 minutes, respectively.

Method II.—In order to eliminate the long time for taking readings necessitated in Method I, calculations were made using the average time between the respective times of maximum color development. In the case of the acetate mixtures described above, the absorbances and the absorptivities were all taken at 24 minutes. In analysis of cholesterol-lanosterol mixtures, the average time of 26 minutes was used.

Calculation by Method I was found to give satisfactory results in all cases (Table III and IV). Method II gave satisfactory results for cholesterol, lanosterol and cholesteryl acetate, but for lanosteryl acetate, the results were much less precise than the results obtained by Method I. The two methods of calculation gave fairly consistent results when whole wool fat was analyzed for combined cholesterol and isocholesterol (as cholesteryl acetate and lanosteryl acetate).

DISCUSSION

In the determination of maximum color development under controlled temperature, it was found that there was a wide variation in the time necessary for maximum color development of cholesterol, lanosterol and their acetate esters (Fig. 2). Cholesteryl acetate develops its maximum in approximately 18 minutes, while free cholesterol develops its maximum color in about 24 minutes. As previously found by other investigators (9), the absorbance of the cholesteryl acetate color complex was about 20% greater than the absorbance produced by an equivalent amount of cholesterol. On the other hand, lanosteryl acetate was found to require approximately 30 minutes for maximum color development, while free lanosterol required only 28 minutes. As with cholesterol, the absorbance of the lanosteryl acetate color complex was found to be about 20% greater than the absorbance of an equivalent amount of lanosterol. Lederer and Tchen (7) had reported a difference of only 5% for lanosterol and lanosteryl acetate. This variation in time and extent of color development was found to be significant in the determination of the amounts of esterified cholesterol and lanosterol in mixtures of their acetates. The absorbances taken at an average time (24 minutes) appeared to be less accurate,

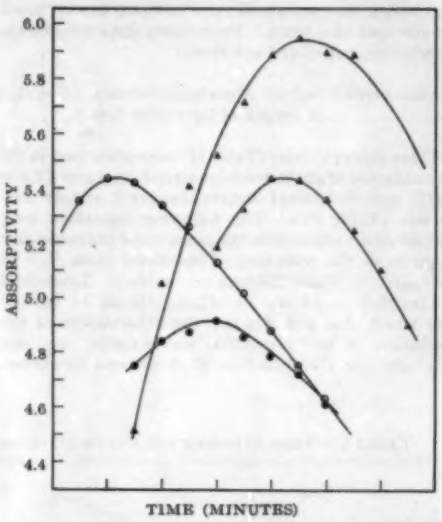

Fig. 2.—Plot showing the relationship of time to absorptivities of cholesterol, lanosterol, cholesteryl acetate and lanosteryl acetate. ● Cholesterol, 630 mμ; ○ Cholesteryl acetate, 630 mμ; ▲ Lanosterol, 550 mμ; Δ Lanosteryl acetate, 550 mμ.

for lanosteryl acetate in particular, than the absorbances taken at 18 minutes for cholesteryl esters (wavelength 630 mμ) and at 30 minutes for lanosteryl acetate (wavelength 550 mμ) (Table IV). The difference of time for color development of unesterified cholesterol and lanosterol is much less, 4 minutes, and therefore, an average time (26 minutes) may be used without a great loss of precision (Table III, Fig. 3).

In order to determine the reliability of the analytical method on unknown samples of wool fat, these samples were analyzed, and the content of cholesterol and isocholesterol esters, calculated as cholesteryl and lanosteryl acetates was determined. After adding known increments of cholesteryl and lanosteryl acetates, the solutions were again analyzed. The concentrations of the new solutions were estimated on the basis of the sum of the pure ester (cholesteryl or lanosteryl acetate) added and the

TABLE IV.—RESULTS OBTAINED ON ANALYSIS OF CHOLESTERYL ACETATE-LANOSTERYL ACETATE MIXTURES AND OF WOOL FAT AND COMPARISON OF RESULTS OBTAINED BY TWO METHODS OF CALCULATION

Sample	Cholesteryl Acetate Taken[a]	Found[b], % Method I[c]	Found[b], % Method II[c]	Lanosteryl Acetate Taken[a]	Found[b], % Method I[c]	Found[b], % Method II[c]
148-A[d]	0.0414	98.1	98.5	0.0274	102.5	107.1
148-B[d]	0.0505	99.1	100.6	0.0302	104.6	106.4
148-C[d]	0.0252	101.3	101.3	0.0549	102.4	109.7
Average		99.5	100.1	..	103.2	107.8
150-A[e]	0.2367	23.1	23.0	..	16.8	16.8
150-B[e]	0.1720	23.0	23.3	..	16.1	17.6
Average		23.1	23.1	..	16.4	17.2

[a] The concentration of sterol (or wool fat) in the color solution expressed as Gm./L.
[b] Percentage of known amount of component, found on analysis of acetate mixtures. For wool fat, percentage of cholesterol and isocholesterol esters in wool fat, calculated as cholesteryl or lanosteryl acetates.
[c] See text. [d] Known mixtures of acetate esters. [e] Wool fat.

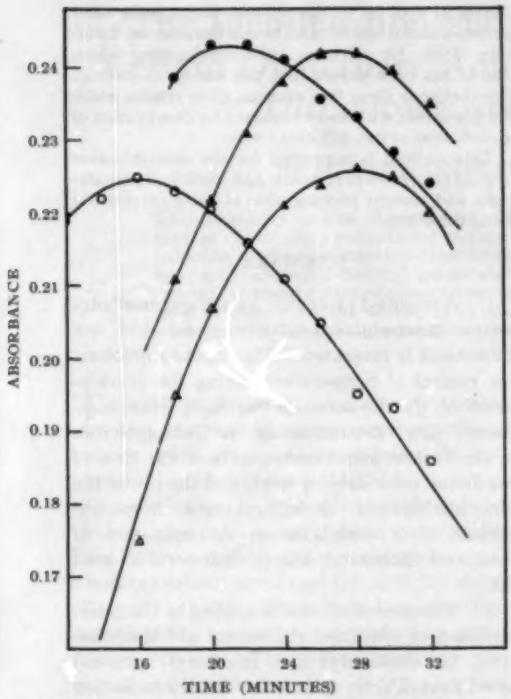

Fig. 3.—Plot showing the relation of time to the absorbances of sample runs of wool fat and wool fat unsaponifiable at 550 and 630 mμ. ○ Wool fat, 630 mμ; Δ Wool fat, 550 mμ; ● Wool fat, unsap., 630 mμ; ▲ Wool fat unsap., 550 mμ.

TABLE V.—RESULTS OF ANALYSIS OF WOOL FAT PLUS ADDED AMOUNTS OF CHOLESTERYL AND LANOSTERYL ACETATES

Sample	Cholesterol Esters Taken[a]	Cholesterol Esters Found[b], %	Isocholesterol Esters Taken[a]	Isocholesterol Esters Found[b], %
150-A-2	0.0647	100.9	0.0507	101.4
150-A-3	0.0748	102.0	0.0617	104.5
150-A-4	0.0697	103.7	0.0563	101.6
150-A-5	0.0596	102.4	0.0452	97.3
150-B-2	0.0446	99.8	0.0322	103.9
150-B-3	0.0496	100.8	0.0387	100.8
150-B-4	0.0547	102.0	0.0442	104.3
150-B-5	0.0597	100.8	0.0496	102.4
Average		101.5		102.0

[a] The estimated concentration of solution, on basis of amount of cholesteryl and isocholesteryl esters (calculated as cholesteryl and lanosteryl acetates) found in wool fat on analysis, plus added amounts of cholesteryl and lanosteryl acetates, expressed as Gm./L.
[b] Percentage of estimated quantity found on analysis.

amount of the esters calculated to be present on the basis of the original analysis. The results obtained were found to agree with the estimated content of the solutions (Table V). Wool fat unsaponifiables were treated in the same manner with added amounts of cholesterol and lanosterol. The results obtained agreed with the estimated concentrations of the solutions (Table VI).

A further test to determine the reliability of the method for estimating the amount of cholesterol and isocholesterol in wool fat was made by calculating, on the basis of molecular weight, the amounts of free sterols equivalent to the esterified sterols found in wool fat. This value was compared to the amount of free sterols in wool fat estimated on the basis of total unsaponifiable from wool fat and the free sterols found in the unsaponifiable. The calculations indicate that comparable results were obtained for cholesterol by either method of estimation (Table VII). In the case of isocholesterol, the results obtained by estimation of lanosterol equivalent to the lanosteryl acetate found in whole wool fat were somewhat higher than those obtained on the basis of the free lanosterol in wool fat unsaponifiable.

TABLE VI.—RESULTS OF ANALYSIS OF WOOL FAT UNSAPONIFIABLES PLUS ADDED AMOUNTS OF CHOLESTEROL AND LANOSTEROL

Sample	Cholesterol Taken[a]	Cholesterol Found[b], %	Isocholesterol Taken[a]	Isocholesterol Found[b], %
152-C	0.0559	98.3	0.0374	100.4
152-D	0.0657	98.9	0.0436	100.4
154-C	0.0489	100.4	0.0322	102.3
154-D	0.0586	101.2	0.0384	99.8
Average		99.7		100.7

[a] The estimated concentration of solution, on basis of amount of cholesterol and isocholesterol (calculated as lanosterol) found in wool fat unsaponifiables on analysis, plus added amounts of cholesterol and lanosterol, expressed as Gm./L.
[b] Percentage of estimated quantity found on analysis.

TABLE VII.—COMPARISON OF RESULTS OBTAINED BY DETERMINATION OF STEROLS IN WOOL FAT DIRECTLY AND DETERMINATION OF STEROLS IN WOOL FAT UNSAPONIFIABLES

Cholesterol esters, calcd. as acetate	23.0%
Free cholesterol equiv. to acetate	20.8%
Isocholesterol esters, calcd. as lanosteryl acetate	16.5%
Free lanosterol equiv. to lano. acetate	15.0%
Unsaponifiable (49.3% of wool fat)	
Cholesterol	41.6%
Equivalent amt. in whole wool fat	20.5%
Isocholesterol calcd. as lanosterol	28.1%
Equivalent amt. in whole wool fat	13.8%

CONCLUSIONS

The application of the spectrophotometric method of Luddy, *et al.* (10), in the present work was found to require modification. The difficulty in its application lies in the variable rate of development of the color complexes of cholesterol, isocholesterol, and

their esters, and in the rapid deterioration of the complexes after formation.

The rate of development of the color complexes was affected greatly by temperature variation. The earlier workers had allowed the reaction to proceed at room temperature, variations of which gave inconsistent results. The first step in this work was to control the temperature of the reaction mixture, so that the rate for each separate component would be constant. A bath temperature of 30° was selected because it was easier to maintain and the rate of color development was increased in some cases.

Luddy, *et al.*, used the absorptivities of the cholesterol and lanosterol complexes for the calculation of all solutions whether they contained the free sterols or their esters. They inserted a factor in the calculation of combined cholesterol to take into account the 20% greater light absorption by the color complex of cholesterol esters as compared to the complex of free cholesterol. They did not use a factor in calculating combined isocholesterol on the assumption that light was absorbed to the same extent by the color complexes of both combined and free isocholesterol (10). On the contrary, it was found in this work that the lanosteryl acetate complex also absorbed about 20% more light than the free lanosterol complex.

The difference in rate of development of and amount of light absorption by the color complexes of the free sterols and their esters was overcome by determining the absorptivities of the complexes of cholesterol, lanosterol, and their acetates at the wavelength and time of maximum light absorption for the cholesterol complex (630 mμ) and the lanosterol complex (550 mμ). The free sterols, as in wool fat unsaponifiables, were calculated by employing the absorptivities of the cholesterol and lanosterol complexes; and the combined sterols, as in wool fat, by employing the absorptivities of the cholesteryl and lanosteryl acetate complexes. The utilization of different absorptivity values corresponding to the free sterols and their esters takes into account the difference of light absorption by the various color complexes. The variation in rate of development is compensated for by using the absorptivities at the respective times of maximum light absorption.

This procedure may be applied to the determination of combined sterols in wool fat directly, or to the determination of free sterols in wool fat unsaponifiable. The former application has the advantage that no previous treatment is necessary, but apparently this method is less reliable and also requires absorbances of unknown solutions to be observed at two different times. The determination of free sterols in wool fat unsaponifiable, seems to be more accurate and is less difficult to run because the difference of time for maximum color development is less, and it has been shown that the use of an average time between these two maxima gives results which are consistent with those obtained by observation of absorbances at two different times.

This method is suggested for the determination of wool fat sterols in cosmetic and medicinal preparations and current investigation is being carried out along these lines.

SUMMARY

1. A modified procedure for the spectrophotometric determination of cholesterol and isocholesterol is presented. This method involves, the control of temperature during the development of the Liebermann-Burchard color complexes, the determination of absorptivities of the various sterol components at the time of maximum color development, and the use of the absorptivities of cholesteryl and lanosteryl acetate color complexes in determination of combined cholesterol and isocholesterol in wool fat.

2. This procedure can be applied to the determination of combined cholesterol and isocholesterol (as cholesteryl and lanosteryl acetates) in wool fat directly and also to the determination of free cholesterol and isocholesterol in wool fat unsaponifiables.

3. Data are presented showing results obtained on application of the method to both wool fat and wool fat unsaponifiables.

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The Identification and Quantitative Analysis of Certain Quaternary Ammonium Compounds using Ultraviolet Absorption Spectra*

By M. PERNAROWSKI and L. G. CHATTEN

The ultraviolet spectra of four quaternary ammonium compounds, 1-hexadecylpyridinium chloride, *p*-diisobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl ammonium chloride, dodecyl-acetamido-dimethyl-benzyl ammonium chloride, and *Beta*-phenoxy-ethyl-dimethyl-dodecyl ammonium bromide, are reported. The use of the spectral characteristics of these compounds in the identification and analysis of the pure substance are discussed. The application of the ultraviolet procedure to the analysis of commercial preparations containing quaternary ammonium compounds is described.

THE INTRODUCTION of the quaternary ammonium compounds into commerce provided industry with a unique substitute for the toxic phenol-type disinfectants. Their widespread use in various industrial operations has presented to the analyst a challenging problem in separation, identification, and quantitative analysis in such unrelated substances as eggs (1), milk (2), shrimp (3), and beer (4). There have been two basic approaches to the analysis of quaternary ammonium compounds. The first involves the preparation of an insoluble derivative of the quaternary ammonium compound and the second involves titration of some portion of the molecular entity.

Substituted ammonium compounds are capable of reacting with certain anions to form salts which are either insoluble or which may be extrated from the reacting phase by means of organic solvents. Examples of such insoluble salts are the ferricyanides, ferrocyanides, silicotungstates, dichromates, and permanganates. Wilson (4) has described a procedure involving the preparation of the ferricyanide salt of the quaternary ammonium compound. Tilson, Eisenberger, and Wilson (5) showed that these basic type compounds were capable to reacting with ammonium reineckate. By preparing these reineckates and determining their optical crystallographic properties, they were able to identify a large number of quaternary ammonium compounds. By far the most common approach to the analysis of this type of compound has been the preparation of a complex using indicators such as bromophenol blue of bromocresol green (1, 3, 6–12). The procedure involves the coupling of the quaternary ammonium compound with the indicator and determining its color intensity in a suitable photoelectric colorimeter.

Several volumetric procedures have been reported for the determination of quaternary ammonium compounds. Carkhuff and Boyd (13) titrated 1-hexadecylpyridinium chloride using sodium lauryl sulphate solution and methyl yellow indicator.

Pifer and Woolish (14) showed the quaternary ammonium compounds could be titrated in nonaqueous solvents with acetous perchloric acid. Caswell (15) reported that the halogen portion of the molecule could be titrated with silver nitrate solution either electrometrically or with dichlorofluorescein indicator solution.

Experience in this laboratory with ultraviolet absorption spectroscopy appeared to indicate that the spectra of these compounds might offer a means of both qualitative and quantitative analysis for various commercial preparations. While this investigation was in progress, a preliminary report on the absorption spectra of two of the quaternary ammonium compounds discussed in this paper was published by Caswell (16).

EXPERIMENTAL

This study included the following compounds: 1-hexadecylpyridinium chloride (Ceepryn® chloride; cetylpyridinium chloride); *p*-diisobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl ammonium chloride (Phemerol® chloride; benzethonium chloride; pheneride; Hyamine 1622®); dodecyl-acetamido-dimethyl-benzyl ammonium chloride (Nopcocide K®); and Beta-phenoxy-ethyl-dimethyl-dodecyl ammonium bromide (Bradosol®).[1]

The pure crystalline quaternary ammonium compounds were analysed by titration in a nonaqueous solvent prior to use. A sample of the quaternary ammonium compound was weighed, dissolved in 50 ml. chloroform and 2 ml. of 6% mercuric acetate in glacial acetic acid and titrated with 0.05 *N* perchloric

* Received August 5, 1957 from the Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.

[1] Synonyms taken from the manufacturer's literature or *The Merck Index*.

acid in dioxane. Methyl red (0.1% solution in glacial acetic acid) was found superior to crystal violet in these titrations and was, therefore, used in all the analysis. The solvent system described by Pifer and Woolish (14) was found to be equally satisfactory.

All preliminary ultraviolet spectra were obtained by scanning a suitable solution from 200 mμ to 300 mμ on a Cary recording spectrophotometer. After maximum and minimum points had been established, analyses of solutions of quaternary ammonium compounds were conducted using a Beckman DU spectrophotometer.

(a) 1-Hexadecylpyridinium chloride.—The specific extinction coefficient (a_s) of 1-hexadecylpyridinium chloride was calculated from the absorbancy (A_s) value observed for a solution containing 0.025 mg. per ml. One cm. cells were used in all determinations. This value of a_s was used to determine the concentrations of all subsequent solutions prepared in this laboratory. A series of solutions were prepared to contain between 0.01 to 0.05 mg. of the quaternary ammonium compound per ml. From calculations of the molar extinction coefficient (a_m) and from a plot of A_s against the concentration, it would appear that Beer's Law is obeyed. Table I records the results obtained.

Figure 1 shows the ultraviolet absorption spectrum of 1-hexadecylpyridinium chloride. The ultraviolet characteristics of this compound are recorded in Table II. The value of a_m at 259 mμ, the point of peak absorbancy, was approximately 4280. This figure represents the average value found for ten determinations representing two separate sets of solutions. An approximate error of 1% was noted between these two sets of determinations.

(b) p-Diisobutyl - phenoxy - ethoxy - dimethylbenzyl Ammonium Chloride.—The procedure followed was essentially the same as that reported in (*a*). The solutions studied had concentrations ranging from 0.08 to 0.40 mg. of the quaternary ammonium compound per ml. Both the molar extinction coefficient and the absorbancy-concentration graph indicated that Beer's Law was being obeyed. The value of a_m at 269.5 mμ, the point of peak absorbancy, showed only slight variation over the whole absorbancy range of the Beckman DU spectrophotometer. This is shown numerically by the low standard deviation reported in Table II. Figure 2 shows the ultraviolet spectrum of p-diisobutyl-phenoxy-ethoxy-dimethyl-benzyl ammonium chloride.

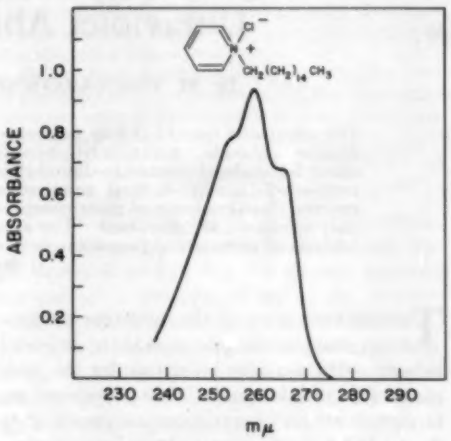

Fig. 1.—The ultraviolet spectrum of 1-hexadecylpyridinium chloride.

TABLE I.—RECOVERY OF QUATERNARY AMMONIUM COMPOUNDS FROM PREPARATIONS OF KNOWN COMPOSITION

Quaternary Ammonium Compound	Purity by Non-aqueous Titration, %	Recovery Spectrophotometric, %	Standard Deviation n = 9
1-Hexadecylpyridinium chloride	99.8	100.4	+1.1
β-Phenoxy-ethyl-dimethyl-dodecyl Ammonium Bromide	100.0	101.0	±1.7
Isododecyl-acetamido-dimethyl-benzyl Ammonium Chloride	100.0	99.1	±2.0
p-Diisobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl Ammonium Chloride	99.4	100.0	±1.0

TABLE II.—ULTRAVIOLET ABSORPTION CHARACTERISTICS OF FOUR QUATERNARY AMMONIUM COMPOUNDS

Quaternary Ammonium Compound	Maxima	Minima	Shoulders	Optimum Concentration mg./ml.	a_m
1-Hexadecylpyridinium chloride	259	...	254–255 264–265	0.04	4280 ± 0.7%
Beta-Phenoxy-ethyl-dimethyl-dodecyl-Ammonium Bromide	268.5 274.5	272.5	263–264	0.20	1285 ± 1.5%
Isododecyl-acetamido-dimethyl-benzyl Ammonium Chloride	263 257.5 269	260 267	252–253	0.50	425 ± 1.9%
p-Diisobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl Ammonium Chloride	269.5 264 275	265 272	257–250 280–281	0.15	1430 ± 0.6%

[a] Values of a_m reported are for the wavelengths underlined. Standard deviations are reported immediately after the a_m value.

This quaternary ammonium compound was one of the two reported in this paper that had been studied by Caswell (16). Table II and Fig. 2 show that this compound has three points of peak absorption. Caswell reported similar observations, indicating that ultraviolet maximum occurred at 264 mμ, 269 mμ, and 274 mμ. Within experimental limits, these results agree well with those observed in this laboratory.

(c) **Beta-Phenoxy-ethyl-dimethyl-dodecyl Ammonium Bromide.**—Solutions were prepared and diluted so that the concentration of the quaternary ammonium compound varied from 0.05 to 0.30 mg. per ml. Absorbancy-concentration graphs and the a_m values at 268.5 indicated that Beer's Law was being obeyed. There appeared to be a slight variation between two sets of solutions. The average a_m value for one set was approximately 1275 while that observed for the other set was approximately 1300. In both cases, however, the standard deviation indicated that either answer was within the experimental limits set by any one series of solutions. The average value is reported in Table II along with other ultraviolet characteristics of this compound. Figure 2 shows the spectral curve for β-phenoxy-ethyl-dimethyl-dodecyl ammonium bromide.

(d) **Dodecyl-acetamido-dimethyl-benzyl Ammonium Chloride.**—The specific extinction coefficient for this compound was determined in the usual way. The concentrations of the solutions varied from 0.1 to 1.0 mg. of the quaternary ammonium compound per ml. Graphical evaluation and a_m values at 263 mμ indicated that Beer's Law was being obeyed. There appeared to be a slight difference in a_m values in those cases where the concentration of the quaternary ammonium compound fell to approximately 0.1 mg. per ml. and exceeded 1.0 mg. per ml. These slight deviations may be expected since the concentrations represent very high and very low absorbancy values. These deviations represent an approximate error of 2% in calculation of percentage recovery from a known preparation.

The ultraviolet characteristics of dodecyl-acetamido-dimethyl-benzyl ammonium chloride are reported in Table II. An ultraviolet spectrum of this compound is shown in Fig. 2. This quaternary ammonium compound, although not reported in tabular form by Caswell (16), was discussed in the text of the paper. He reported that this compound showed the same ultraviolet characteristics as did alkyl-dimethyl-benzyl ammonium chloride and cetyl-dimethyl-benzyl ammonium chloride. Evidently the absorption appears to be characteristic of the benzyl group. There was excellent agreement between the results obtained in this laboratory and those reported by Caswell.

The following general procedure is recommended for estimating the concentration of compounds of the type mentioned in this paper.

Weigh accurately the quaternary ammonium compound and dissolved in sufficient water to make the concentration such that it will be suitable for ultraviolet examination. Dilution of the original solution by the aliquot procedure may be required. Since many quaternary ammonium compounds are marketed in an aqueous solution, dilution to the required concentration may be the only step required. Transfer a portion of the solution to a 1-cm. cell, and using water as the blank, scan the solution

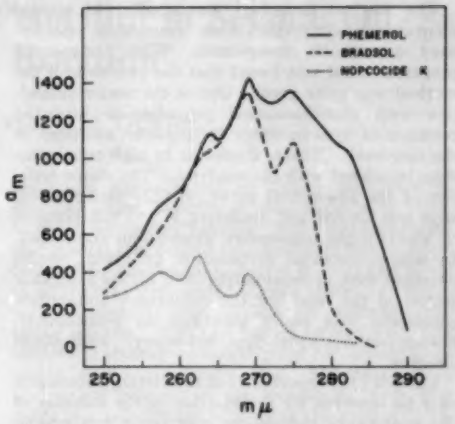

Fig. 2.—The ultraviolet spectra of three quaternary ammonium compounds.

on the Cary recording spectrophotometer from 220 to 300 mμ. Determine the absorbancy from the chart record or by using a Beckman DU spectrophotometer set at the required wave length. By observing the characteristics of the spectra and from the value of a_m obtained for the compound, the quaternary ammonium compound may be identified with some degree of certainty. For the purposes of this paper, the a_m value at peak absorption has been reported. Values for a_m at any other absorption maxima or minima may be calculated for the purposes of identification. Where the original concentration of the quaternary ammonium compound is unknown, a standard should be run under identical conditions to establish a_s values.

DISCUSSION

There appears to be a certain amount of similarity between three of the four spectra. Dodecyl-acetamido-dimethyl-dodecyl ammonium bromide, and *p* - diisobutyl - phenoxy - ethoxy - ethyl - dimethyl-benzyl ammonium chloride both show an absorption maxima at 269 mμ. At this point, only a_m values would serve to distinguish the compounds. In addition, the first and last compounds also show absorption maxima at approximately 264 mμ. Similarly, the second and third compounds show similarity in the region 274 mμ . The actual differences are best seen in Fig. 2. In this figure, a_m values have been plotted against the wave length. It will be noted that the point of greatest difference between β-phenoxy-ethyl-dimethyl-dodecyl ammonium bromide and *p*-diisobutyl-phenoxy-ethyl-dimethyl-benzyl ammonium chloride lies at 275 mμ. This point is, therefore, a key point in positive identification of the quaternary ammonium compound. Experimental errors may give inconclusive results at 269 mμ or at 263 to 264 mμ. No difficulty should be experienced in distinguishing between dodecyl-acetamido-dimethyl-benzyl ammonium chloride and the above mentioned compounds. 1-Hexadecyl-pyridinium chloride presents no problem because its peak absorption occurs at much lower wavelengths.

The method described was applied to several pharmaceutical preparations containing quaternary ammonium compounds. With commercial preparations, it was found that the precision of the method was quite good. One of the major difficulties with pharmaceutical preparations was the presence of dyes in either the aqueous solutions or the tinctures. These, if present in high concentration, interfered with the analysis. The characteristics of the absorption curve, in the cases studied, were not altered and, therefore, it was still possible to identify the quaternary ammonium compound. In one commercial preparation analyzed, results obtained were approximately 30% higher than that stated on the label but the quaternary ammonium compound was easily identified as *p*-diisobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl ammonium chloride.

Caswell (16) reported that interfering substances may be removed by acidification of the solution of the quaternary ammonium compound and extraction of the substance with ether. Another possible approach involves the use of the base line technique. It was found in this laboratory that incorporation of the dye present in the solution into the blank gave satisfactory results. If the dye concentration is not high, the color intensity is low and the quaternary ammonium compound can be determined. The results obtained for a typical preparation are shown in Table III. The results in this Table indicate that an error of 10% in the dye concentration will still lead to satisfactory results. Usually

TABLE III.—ABSORBANCY VALUES FOR COLORED AND UNCOLORED SOLUTIONS OF P-DIISOBUTYL-PHENOXY-ETHOXY-ETHYL-DIMETHYL-BENZYL AMMONIUM CHLORIDE

Determination	Mg./ml. of Compound	Mg. Dye/L. of Solution	Mg. Dye/L. of Blank	A_2
I	0.12	0.00	0.00	0.368
				0.371
II	0.12	5.00	5.00	0.363
				0.380
III	0.12	5.00	4.50	0.374
				0.372

a calibration curve was obtained for the dye using a Cary recording spectrophotometer and the dye concentration in the commercial preparation was thus determined. This was then utilized in preparing a proper dye concentration in the blank. The above method was found to be very satisfactory for laboratory preparations and for several commercial preparations. Two commercial preparations could not be analyzed in this way because of high dye concentration (of the order of 40 mg. per liter in the final dilution).

Not only is the presence of dye a limiting factor, but also the presence of alcohol will cause difficulty. In this case, the problem is less complex and with proper dilution (usually with 95% alcohol) Beer's Law is valid. This was shown to be the case for *p* - diisobutyl - phenoxy - ethoxy - ethyl - dimethyl-benzyl ammonium chloride, 1-hexadecylpyridinium chloride, and dodecyl-acetamido-dimethyl-benzyl ammonium chloride. In the presence of alcohol, however, peak absorbancies reported in water are not valid. These must be established on the basis of the preparation being analyzed. Slight shifts in peak absorption points and changes in a_m values were noted for the three preparations listed above. As an example, *p*-diisobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl ammonium chloride showed four absorption maxima (λ max. = 263, 269, 276, 283 mμ) in approximately 50% ethanol. It would appear that the shoulder at 280–281 mμ in aqueous solution becomes a peak in alcoholic solution. The differences for the other quaternary ammonium compounds were found to be less pronounced. Again, the first of these three quaternary ammonium compounds listed above was studied in a colored, aqueous alcohol solution. By using the technique described above, it was found that a linear relationship existed between the absorbancy of the solution and its concentration.

In recent months, Crane (17) showed that sodium tetraphenylboron formed an insoluble derivative with certain quaternary ammonium compounds. This is being investigated in this laboratory at the present time. Preliminary results show that not only quaternary ammonium compounds but also other compounds having a basic nitrogen may be separated from mixtures of substances not having this property.

The most plausible approach to commercial preparations containing quaternary ammonium compounds would appear to be extraction of the substances into a nonaqueous solvent and subsequent titration with perchloric acid in dioxane. This approach was investigated and found to give precise results. However, the accuracy of the method was not as good as expected. Only about 95% of the active ingredient could be extracted using conventional techniques.

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The Assay of Procaine Penicillin in Sesame Oil by Optical Rotation*

By B. A. PAPPAS and J. D. DUERR

The extraction of Procaine Penicillin from a suspension containing sesame oil and aluminum monostearate using chloroform and formamide with subsequent assay by optical rotation is described. It is also compared to an assay in present use involving the solution of the suspension in hot chloroform and subsequent assay by optical rotation.

OF NECESSITY CONTROL PROCEDURES in the pharmaceutical industry must be rapid and yet accurate. Most of the assays have been designed for quick determination and one of the quickest is optical rotation. Penicillin salts in general can be assayed by optical rotation after finding a suitable solvent. This method, for control purposes while formulating and filling, is very rapid and accurate.

When the formulation contains a vehicle that is difficult to remove from the active material, or contributes cloudiness or haziness to the solution, the accuracy of the assay procedure invariably suffers. Filtering almost any solution containing a solvent commonly used for the determination of optical rotation will result in erratic results, usually on the high side. The solvents commonly used for the assay of procaine penicillin when applied to oil suspensions containing aluminum monostearate either did not dissolve the procaine penicillin or gave extremely hazy solutions even after heating and filtering. The solvent generally used is chloroform. The extracts are usually heated and filtered. The present experiments were undertaken in order to find a solvent or solvent mixture that would dissolve the Procaine Penicillin, free the oil and aluminum monostearate and leave a clear solution ready for measurement by optical rotation. At the same time the intention was to eliminate the deviation between filtered and nonfiltered solutions, the deviation between filtered samples themselves, and the deviation due to different heating conditions used to dissolve the sample suspension.

EXPERIMENTAL

Assay of a Standard Suspension of Procaine Penicillin in Oil.—A suspension was made to contain exactly 300,000 units of procaine penicillin per ml. with a specific gravity of 1.0. The vehicle was 2% aluminum monostearate in sesame oil. Five individual 3-Gm. samples of suspension were transferred to separatory funnels. Fifty ml. of formamide (Special Decolorized Charles Pfizer Grade manufactured by Matheson, Coleman & Bell) and 25 ml. of chloroform were added to each separatory funnel and the mixture was shaken for approximately thirty seconds. The two phases formed were then allowed to separate completely and the bottom formamide layer was optically clear. Measurements of the optical rotation of the formamide layer were made in a 1-decimeter tube using the sodium D line illumination. The results are shown in Table I.

TABLE I.—ANGLE OF ROTATION OF FIVE 300,000 UNITS/CC. STANDARDS

Sample	Total Units	Angle°
1	900,000	2.64
2	900,000	2.65
3	900,000	2.64
4	900,000	2.63
5	900,000	2.64

The conclusion, therefore, was that an average angle of rotation of 2.64° would be obtained when 900,000 units of the above standard procaine penicillin in sesame oil were treated using this procedure.

The Effect of Varying the Concentration of Sesame Oil and Aluminum Monostearate.—Samples of the standard procaine penicillin containing 900,000 units each were transferred to separatory funnels. Diluent containing sesame oil and aluminum monostearate was added to each sample. The volume of diluent ranged from 1.0–3.0 ml. The assay procedure was repeated and the formamide solutions were read in a 2-decimeter tube. The effect of varying concentrations of aluminum monostearate and sesame oil on the angle of rotation is shown in Table II. We concluded, therefore, from the results in Table II that small variations in concentration of diluent had no serious effect on the optical rotation since the difference between the highest and lowest concentration was only 1.34%.

TABLE II.—EFFECT OF CONCENTRATION OF SESAME OIL AND ALUMINUM MONOSTEARATE ON ANGLE OF ROTATION

Units Procaine Penicillin	Vol of Added Oil and A.M.S., cc.	Angle of Rotation
900,000	1	5.15
900,000	1.5	5.20
900,000	2.0	5.20
900,000	3.0	5.22

Reproducibility of Assay Using Different Weights of Sample.—Samples of a standard suspension of 300,000 units/ml. ranging from 0.9306 Gm. to

* Received June 26, 1957 from Chas. Pfizer & Co. Inc., Process Development Laboratory, Pharmaceutical Production and Packaging Department, Brooklyn, N. Y.

TABLE III.—REPRODUCIBILITY OF ASSAY USING DIFFERENT WEIGHTS OF SAMPLE[a]

Wt. of Sample, Gm.	Units Introduced	Angle of Rotation	Units Recovered	Units/ml.	Deviation in Thousands
0.9306	279,180	0.80	272,727	293,060	−6.6
1.5519	465,570	1.40	477,272	307,540	+7.8
1.7596	527,880	1.54	525,000	298,400	−1.3
2.5786	773,580	2.25	751,894	291,500	−8.2
2.9130	873,900	2.55	869,318	298,400	−1.3
3.0000	900,000	2.64	900,000	300,000	0
3.0000	900,000	2.65	903,000	301,000	+1.3
3.0000	900,000	2.64	900,000	300,000	0
3.0000	900,000	2.63	897,000	299,000	−0.7
3.2450	973,500	2.88	981,000	302,000	+2.3
3.7358	1,120,740	3.34	1,138,636	304,600	+4.9
3.8280	1,148,400	3.38	1,152,272	301,000	+1.3

[a] Theoretical Potency = 300,000 units/ml.; Average Assay = 299,708 units/ml.; Standard Deviation of Assay = 4,175 units/ml.

3.8280 Gm. were assayed using the same procedure. The standard procaine in oil suspension was prepared as follows: An amount of bulk procaine penicillin of known potency was mixed with sesame oil containing 2% aluminum monostearate to give a theoretical potency of 300,000 u/ml. The standard average angle of 2.64° was used to calculate total units per ml. recovered by assay. The results are shown in Table III.

Assay of an Unknown.—Five gallons of a production lot were set aside in the laboratory and used as an unknown before the potency was confirmed. All subsequent reference to an unknown pertains to this 5-gal. sample. Three samples were assayed and their potency calculated using an optical rotation of 2.64° equivalent to 300,000 u/ml. The results are shown in Table IV.

TABLE IV.—ASSAY OF UNKNOWNS

Sample	Theoretical Units Introduced	Units Recovered by Assay
1	301,000	301,200
2	710,000	718,000
3	886,000	889,000

Reproducibility of Assay.—Twenty samples from the unknown mixture of procaine Penicillin sesame oil were assayed by two technicians using 3.0-Gm. samples. The standard angle of 2.64° was used to calculate units per ml. of procaine penicillin. The results are shown in Table V.

Biological Assay of the Same Unknown.—The unknown used in the previous experiments was submitted for the microbiological assay of procaine penicillin. The results are shown in Table VI.

Assay Using Chloroform as the Solvent.—Ten samples were taken from the same unknown and each transferred to a 50-ml. volumetric flask. The suspension was then shaken with chloroform and the flask brought to volume with chloroform. A very cloudy solution developed. The flasks were then immersed in hot water until the solution cleared.

TABLE V.—COMPOSITE OF RESULTS OF TWO TECHNICIANS[a]

Sample	Angle of Rotation	Potency units/ml.	Deviation in Thousands	(Deviation in Thousands)^2
1	2.60	295,400	+3.5	12.25
2	2.55	289,700	−2.2	4.84
3	2.57	292,000	+0.1	0.01
4	2.59	294,300	+2.4	5.76
5	2.57	292,000	+0.1	0.01
6	2.58	293,200	+1.3	1.69
7	2.53	287,500	−4.2	17.64
8	2.57	292,000	+0.1	0.01
9	2.58	293,200	+1.3	1.69
10	2.56	290,900	−1.0	1.00
11	2.60	295,400	+3.5	12.25
12	2.56	290,900	−1.0	1.00
13	2.56	290,900	−1.0	1.00
14	2.56	290,900	−1.0	1.00
15	2.56	290,900	−1.0	1.00
16	2.56	290,900	−1.0	1.00
17	2.57	292,000	+0.1	0.01
18	2.57	292,000	+0.1	0.01
19	2.56	290,900	−1.0	1.00
20	2.56	290,900	−1.0	1.00

[a] Average Potency = 291,900 units/ml., Standard Deviation = 1,790 units/ml., 2 σ Limits = 288,320 to 295,480 units/ml., 95% Confidence interval observed value ± 1.22%. 99% Confidence interval = observed value ±1.84%.

TABLE VI.—RESULTS OF BIOLOGICAL ASSAY[a]

Sample	Potency Units/ml.	Deviation in Thousands	(Deviation in Thousands)^2
1	285,000	+1	1
2	285,000	+1	1
3	280,000	−4	16
4	295,000	+11	121
5	280,000	−4	16
6	290,000	+6	36
7	300,000	+16	256
8	280,000	−4	16
9	280,000	−4	16
10	285,000	+1	1

[a] Average Potency = 284,000 units/ml., Standard Deviation = 6,900 units/ml., 2 σ Limits = 270,200 to 297,800 units/ml. 95% Confidence interval observed value ±5.21%. 99% Confidence interval observed value ±7.28%.

TABLE VII.—POTENCY OF UNKNOWN PROCAINE PENICILLIN IN SESAME OIL USING $CHCl_3$ AS SOLVENT AND FILTERED BEFORE READING

Sample	Potency Units/ml.	Deviation	(Deviation in Thousands)[2]
1	311,592	+ 2.3	5.29
2	308,210	− 1.1	1.21
3	314,874	+ 5.6	31.40
4	316,540	+ 7.2	51.80
5	321,538	+12.2	148.84
6	304,878	− 4.4	19.40
7	304,878	− 4.4	19.40
8	304,878	− 4.4	19.40
9	301,546	− 7.8	60.84
10	308,210	− 1.1	1.21
11	304,878	− 4.4	19.40

Average Potency = 309,275 units/ml.; Standard Deviation = 5,850 units/ml.
2 σ Limits = 297,575 to 320,975 units/ml., 95% Confidence interval = Observed value ±3.71%, 99% Confidence interval = Observed value ±5.67%.

TABLE VIII.—POTENCY OF UNKNOWN PROCAINE PENICILLIN IN SESAME OIL USING $CHCl_3$ AS SOLVENT AND READ UNFILTERED[a]

Sample	Potency units/ml.	Deviation in Thousands	(Deviation in Thousands)[2]
1	299,880	−3.0	9.00
2	301,546	−1.4	1.96
3	308,210	+6.3	3.97
4	306,544	+3.6	3.97
5	304,878	+2.0	4.00
6	299,880	−3.0	9.00
7	299,880	−3.0	9.00
8	301,546	−1.4	1.96
9	301,546	−1.4	1.96
10	299,880	−3.0	9.00
11			

[a] This particular sample of procaine penicillin in oil was clear enough to read without having to resort to approximation on the part of the technician. Average Potency = 302,905 units/ml.
Standard Deviation = 2,240 units/ml.
2 σ Limits = 298,425 to 307,385 units/ml., 95% Confidence = 1.47%, 99% Confidence = 2.21%.

Care was taken that the chloroform solution was not brought to the boiling point. The solutions were then cooled until they returned to a 50-ml. volume. The angle of rotation of each solution was read using the sodium D illumination both filtered and unfiltered. The potency was determined using the following formula:

$$\frac{\alpha \times \text{Volume of Solution} \times \text{S.G.} \times 1008}{(\text{Units/mg}) / ([\alpha]_D^{25^\circ} \times \text{Wt.})} = \text{Units/ml.}$$

Procaine Penicillin where $[\alpha]_D^{25^\circ} = 102$; S.G. = 1.0; Wt. = 3.0 Gm., Vol. = 50 ml. and Units/mg. Procaine Penicillin = 1008. The results are shown in Table VII and VIII.

SUMMARY

1. The potency of procaine penicillin in sesame oil suspensions can be determined by using formamide and chloroform for extraction and subsequent measurement of optical rotation. The average potency comes closer to the average reported by the microbiological method than the result obtained by determining the potency by optical rotation in straight heated chloroform.

2. The average deviation and standard deviation of this assay is far lower than those obtained using either the microbiological assay or the chloroform assay.

3. The results show that the difference obtained between filtered and unfiltered chloroform solutions is very great. Average and standard deviations are higher when the chloroform solution is filtered.[1]

4. Extraction using formamide and chloroform gives results that approach theoretical potencies of procaine penicillin in sesame oil suspensions much closer than those obtained by either microbiological assay or the use of heated chloroform.

5. Since one cannot know in advance whether the chloroform solution has to be filtered before reading the optical rotation of the solution, it would seem advisable to adopt a method that eliminates the necessity of filtering at any time.

TABLE IX.—A COMPARISON OF DEVIATIONS AND PER CENT CONFIDENCE OF THE THREE METHODS OF ASSAY

Methods	Average Potency in units/ml.	Standard Deviation in units/ml.	2 σ limits in units/ml.	95% Confidence interval	99% Confidence interval
Formamide chloroform	291,900	1,790	295,480 288,320	±1.22	±1.84
Chloroform (filtered)	309,275	5,850	320,975 297,575	±3.71	±5.67
Bioassay	284,000	6,900	297,800 270,200	±5.21	±7.28
Chloroform (unfiltered)	302,905	2,240	307,385 298,425	±1.47	±2.21

[1] Much weight must be given to conclusion 3. The effect of filtration is not made clear by separate analysis of filtered and unfiltered results. In reality the effect of filtration is randomly distributed, since the practice of necessity, is to filter unreadable samples. This practice leads to very large deviations as can readily be seen from casual inspection of the separate results.

The Effects of Certain Carbon Sources on The Growth of *Claviceps litoralis**

By W. T. GLOOR, Jr.† and H. W. YOUNGKEN, Jr.‡

Comparative growth and dry weight yields of *Claviceps litoralis*, Kawatani, grown in synthetic media with nine different carbon sources are reported. A five per cent mannitol concentration, which served as the carbon source for mycelial growth, produced best results. Glucose, mannose and levulose produced good growth and weight; whereas maltose, sorbitol, glycerol, sorbose, and xylose gave poor weight yields.

GROWTH OF THE ERGOT FUNGUS *Claviceps purpurea* (Fries) Tulasne has been accomplished on artificial culture under various conditions by numerous workers (1–10). However, the growth obtained under these conditions produced either no alkaloids or insignificant amounts of them when compared with that produced in the natural sclerotium of *Claviceps*. Bonns (1) and Kirchhoff (2) reported the formation of "sclerotial tissue" in artificial cultures but this result was questioned by McCrea (3) who contended it was not true sclerotium but a "pseudosclerotium." Abe (9) suggests that pigmented sclerotial tissue is a necessary factor for the biosynthesis of ergot alkaloids in culture. Abe's contention has been in part supported by Sim and Youngken (12) and by Loo and Lewis (11) using two different experimental approaches.

During a screening program of various species and strains of *Claviceps* undertaken in this laboratory, the species, *Claviceps litoralis*, Kawatani, obtained from Dr. T. Kawatani (13, 14) was found to produce purplish brown pigmented, "pseudo-sclerotial" tissue in artificial media (12). The pigment closely resembled that found in natural rye ergot. The fact that no alkaloids were formed led us to believe that some precursor(s) or growth conditions necessary for the production of alkaloids might be lacking. Since it is a well known phenomenon that many fungi respond in different ways to subtle changes in carbon sources during growth on artificial culture, it appeared of interest to investigate several of these conditions with respect to the growth, pigmentation and alkaloid production of *C. litoralis*, Kawatani, grown on synthetic media. This report describes the results obtained by using nine different carbon sources.

* Received June 11, 1957 from the College of Pharmacy, University of Washington, Seattle, Wash.

This research has been supported in part by funds from the State of Washington Fund for Medical and Biological Research and the American Foundation for Pharmaceutical Education.

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EXPERIMENTAL

Culture Method.—Previous work on the growth studies of *C. litoralis*, Kawatani (13, 14) indicated that maltose might not be as satisfactory a carbon source as other compounds. Therefore, in preliminary work of this study 5% mannitol was used as the carbon source in a modified yeast extract-peptone medium (5) described in Table I. Mannitol produced better growth results during preliminary studies. This alcohol has also been isolated from ergot sclerotia both in free form and from Claviceps in, a trisaccharide occurring in the sclerotia.

TABLE I.—MODIFIED YEAST EXTRACT-PEPTONE MEDIUM

Yeast extract	12.50 Gm.
Peptone	1.25 Gm.
$MgSO_4.7H_2O$	1.25 Gm.
KH_2PO_4	2.50 Gm.
Mannitol	50.00 Gm.
Distilled Water *q.s. ad*	1.00 L.

Eight other carbon compounds, namely, sorbose, mannose, levulose, glucose, maltose, sorbitol, xylose, and glycerol were therefore selected and used in a molar strength corresponding to a 5 per cent mannitol solution (0.275 *M*). Each compound was made up into a separate medium using the same amounts of peptone, yeast extract, magnesium sulfate, and potassium phosphate as described in Table I.

Erlenmeyer flasks of 500-ml. capacity were employed as culture flasks with each flask containing 100 ml. of medium. Five milliliters of a mycelium suspension were inoculated into each flask of test medium.

The inoculum was prepared by first inoculating the fungus, which had been maintained on agar slants, into yeast extract-peptone medium containing 5% mannitol. This preparation was allowed to grow on a reciprocal laboratory shaker operating at two hundred $1^1/_2$-inch strokes per minute. At the end of four weeks the contents of the flask were poured into a sterilized steel Waring Blendor cup and blended into a fine even suspension. Five milliliters of the suspension were inoculated into each flask of test medium after which the flasks were placed on the mechanical shaker. After a 15-day growth period, (see Fig. 1) the flasks were harvested by filtering the growth through tared sintered glass crucibles and thoroughly washed with distilled water to remove all adhering constituents of the medium. The growth was dried as much as possible using a Büchner flask and vacuum and then placed in a laboratory lyophilizer to remove com-

pletely all remaining moisture. The crucibles were then weighed and the dry weights calculated (Table II, Fig. 2).

The spent culture liquids and the lyophilized tissues were tested for the presence of alkaloids using the procedure reported by Sim and Youngken (12). However, no ergot alkaloids could be detected in any of the different culture liquids or tissues.

No attempt was made to compare the results obtained above with those reported by other workers (1–10) for other species of *Claviceps* because many factors differ in each case. Different strains and species have been used as the test organism and a wide variety of media and culture conditions have been reported. Nevertheless, the type of growth obtained with mannitol was very similar to the results reported by Sim and Youngken (12) and consisted of pigmented "sclerotial" tissues and unpigmented mycelial tissues and contained large amounts of fat. This production of fatty material closely resembles the situation found in natural sclerotia; however, the fungus failed to produce any alkaloids under these artificial conditions.

The amounts of mycelial growth which were obtained using glucose, mannose, and levulose as the individual carbon sources are comparable to those obtained with mannitol. However, fungal tissues grown on these three carbohydrates were a light brownish color instead of the purplish brown color which was produced by mycelia grown on mannitol. Although some fatty material was also produced by the organism growing on these three sugars, it did not compare quantitatively with the amount of lipid material produced by the *Claviceps litoralis* grown on mannitol.

Maltose as a carbon source produced only half as much growth as was produced by the control, mannitol. This was to be expected as this fact was shown by previous work with this particular organism (12). The remaining four carbon compounds, sorbitol, glycerol, sorbose, and xylose, could be considered very poor carbon sources under the experimental conditions used. The mycelium pellets grown on maltose were a dirty brown color while the pellets grown on the latter four compounds were small, tough, dark brown pellets. The production of lipid material by the tissues grown on the above five carbon sources was also noticeably small by comparison.

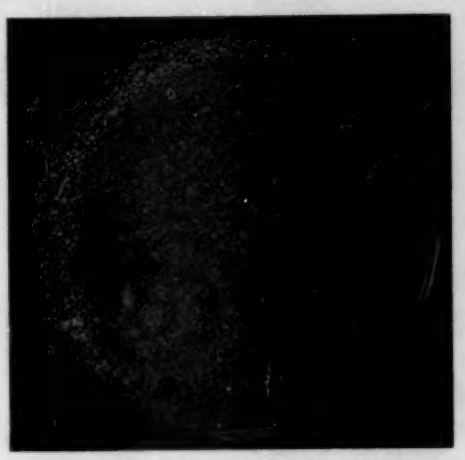

Fig. 1.—Typical mycelial pellets of *Claviceps litoralis* grown under shaking conditions on 5 per cent mannitol medium for fifteen days. (×1.)

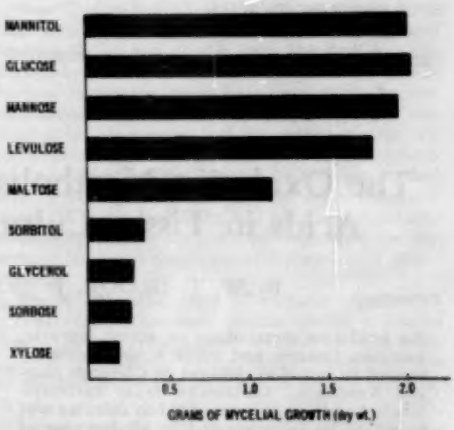

Fig. 2.—Comparison of dry weights of *Claviceps litoralis* mycelial growth on different carbohydrates

TABLE II.—COMPARISON OF DRY WEIGHTS OF CLAVICEPS LITORALIS MYCELIAL GROWTH ON DIFFERENT CARBOHYDRATES

Test Carbohydrate	Concentration, %[a]	No. of Flasks	Av. Dry Wt., Mycelium/ Flask, Gm.	Standard Deviation of Mean[b]	Relative, Dry Wt. of Growth, %
Mannitol[c]	5	6	2.0120	±0.0566	100
Glucose	5	7	2.0174	±0.0239	100.27
Mannose	5	7	1.9404	±0.0413	96.44
Levulose	5	7	1.7975	±0.0391	89.34
Maltose	10	7	1.1397	±0.0289	56.65
Sorbitol	5	6	0.3343	±0.0186	16.62
Glycerol	2.5	6	0.2722	±0.0109	13.53
Sorbose	5	7	0.2583	±0.0045	12.84
Xylose	4	7	0.1602	±0.0088	7.96

[a] Calculated on the molar concentration of 5 per cent mannitol solution (0.275 *M*).

[b] Standard deviation of the mean = $\pm\sqrt{\frac{\Sigma d^2}{n + (n-1)}}$

[c] Control.

SUMMARY

A comparative growth study of *C. litoralis*, Kawatani, on nine different carbon sources has been described.

Of the nine carbon sources tested, mannitol, glucose, mannose, and levulose produced abundant and comparable amounts of growth; maltose produced only fair growth while sorbose, sorbitol, glycerol, and xylose produced poor to very poor results.

The tissue grown on mannitol produced some purplish pigmentation resembling that of natural ergot while the fungal material grown on the other compounds produced only brownish to dark brown pigmentation.

It was noted that the mannitol-grown mycelium produced large amounts of fat-like material while the tissues grown on other carbohydrates produced only small amounts. The production of large amounts of fats and sterols is a condition also found in natural ergot.

While none of the fungal tissues grown on the various carbon sources, including mannitol, produced any detectable amounts of alkaloids, mannitol could be considered the most desirable carbon source since mycelial tissues grown on it produced by comparison more characteristics resembling the natural sclerotia than did the mycelia grown on the other carbon compounds.

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The Oxidative Metabolism of Certain Krebs Cycle Acids in Tissue Cultures of *Claviceps litoralis**

By W. T. GLOOR, Jr.† and H. W. YOUNGKEN, Jr.‡

An oxidative metabolism of acetic, pyruvic, succinic, fumaric and malic acids is demonstrated in mycelial cultures of *Claviceps litoralis* Kawatani. Carbon-14 from carboxyl-labeled sodium acetate added to cultures was found to be incorporated in all but one of these acids (succinate). However no detectable amounts of tricarboxylic acids could be isolated from the mycelial tissues. It is concluded that a complete Krebs cycle probably does not exist in the fungus when it is grown under the synthetic culture conditions described.

SINCE THE elucidation by Krebs (1) of the tricarboxylic acid cycle in mammalian tissues, many attempts have been made to show the presence of this metabolic cycle as an oxidative pathway in plants and microorganisms. Recently Millerd *et al.* (2) have shown that the complete Krebs cycle exists in mung bean seedling mitocondria. This is the first work to demonstrate the presence of the complete cycle in higher plants. More recently, Krebs *et al.* (3), Krampitz *et al.* (4–6) and others (7–9) have reported conclusive evidence for the existence of the entire cycle in microorganisms.

In view of the importance of the TCA cycle in the oxidative metabolism of carbohydrates in various organisms, it was thought that if such a pathway could be demonstrated in a *Claviceps spp.*, this might serve as a starting point for further attempts to elucidate a biosynthetic pathway of alkaloid formation in *Claviceps*. In the studies cited above, manometric data have been an important part of the evidence presented. However, preliminary manometric studies proved quite disappointing because of the high rate of endogenous respiration of the *C. litoralis* tissues. When attempts to overcome this problem proved unsatisfactory, it became necessary to use some other method in an attempt to demonstrate the

* Received June 11, 1957 from the College of Pharmacy, University of Washington, Seattle, Wash.
This research has been supported in part by funds from the State of Washington Fund for Medical and Biological Research and the American Foundation for Pharmaceutical Education.
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presence of a TCA cycle. This report describes the culturing of *Claviceps litoralis*, Kawatani, on carbon-14 carboxyl-labeled sodium acetate and the subsequent isolation of carbon-14 labeled TCA cycle components.

EXPERIMENTAL

Culture Method—Inoculation cultures of *Claviceps litoralis* mycelial tissues were made by inoculating liquid medium by means of an inoculating needle, from a stock culture agar slant. The liquid medium used in this study consisted of 5% mannitol, 1.25% yeast extract, 0.25% KH_2PO_4, 0.125% peptone and 0.125% $MgSO_4.7H_2O$. These cultures, which consisted of 100 ml. of inoculated medium in a 500-ml. Erlenmeyer flask, were continuously shaken on a reciprocal shaker operating at 200 strokes per minute. After four weeks the cultures were removed and the contents of the flask whipped into a fine suspension in a sterile Waring Blendor cup. This suspension was used to inoculate other flasks of yeast-extract-peptone medium to produce the growth used in this study. Five milliliters of the suspension were inoculated into each flask of 100 ml. of medium. These flasks were then allowed to grow on the reciprocal shaker for 15 days after which the growth was filtered and washed clean of adhering medium constituents with distilled water. Under these conditions the growth consisted mainly of small, roughly-spherical pellets approximately 1-3 mm. in diameter.

The pellets of fungal growth material were then handled in two ways. One group, approximately a third of the material, was not treated in any way and will be referred to as "freshly harvested material" or "unstarved material." The organic acids were extracted and isolated from this material as described below.

The remainder of the mycelial pellets, usually about 200 grams (wet weight), was placed in 1 liter of 0.1 *M* phosphate buffer solution (pH 6.8). This mixture was aerated vigorously in a covered 4-liter beaker for 3 days. During the 3-day period the buffer solution was replaced with fresh buffer solution at 4- to 12-hour intervals. At first, the aerating solution was checked several times every day for possible bacterial contamination; but this practice was discontinued later when no evidence of contamination appeared. The pellets treated in the above fashion will be referred to as "starved" mycelium.

The "starved" material was divided into two groups. One part of the mycelial material was given no additional treatment but extracted for organic acids as described below. The second portion of the "starved" material was treated with carbon-14 carboxyl-labeled sodium acetate. To carry out this treatment an arrangement of apparatus was used which was similar to that employed by Djao (10) (Fig. 1). A 500-ml. Erlenmeyer flask was fitted with an inlet tube connected to a bubble trap containing barium hydroxide solution and an outlet tube which led to a series of two bubble traps containing standardized barium hydroxide solution. The second bubble trap was connected to a vacuum flask. Into the Erlenmeyer flask were placed 30 Gm. (wet weight) of "starved" mycelium pellets,

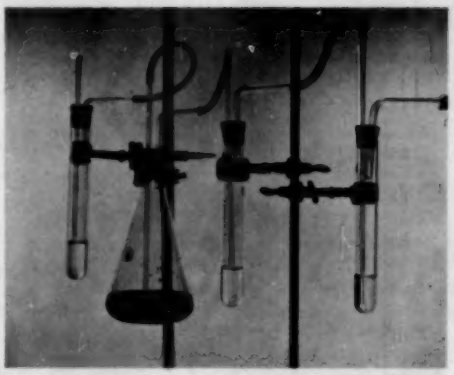

Fig. 1.—Apparatus for collection of carbon-14 labeled carbon dioxide released by *Claviceps litoralis* from carboxyl labeled sodium acetate

116 ml. of 0.1 *M* phosphate buffer solution and 4 ml. of labeled sodium acetate solution, 0.3 *M* (described below). When a vacuum was applied to this system, carbon dioxide-free air was admitted through the inlet tube into the flask, agitating the mixture and flushing the carbon dioxide from the flask into the barium hydroxide solution in the bubble traps. The barium carbonate precipitate was used for the measurement of radioactivity. The mycelium was treated in this manner and the carbon dioxide collected over a period of 2 hours. Then the mycelium was removed from the solution by filtering, washed free of acetate and the organic acids extracted. After the individual acids had been separated and identified, they were oxidized separately as described later and their radioactivity content determined.

Tracer Material and Technique—Carbon-14 carboxyl-labeled sodium acetate having a labeled activity of 2 μc per millimole was diluted with sufficient nonradioactive sodium acetate and dissolved in distilled water to give a 0.3 *M* acetate solution. When 1 ml. of this solution was diluted to 30 ml., the final concentration of acetate was 0.01 *M* and the activity was approximately 25,000 counts per minute. Specific activity(counts/minute/mg. of carbon) of this solution was 3.35×10^4.

A convenient method of oxidizing samples in preparation of isotope assays is by the wet oxidation method (11) which was employed in this study. The carbon dioxide is absorbed in a 0.25 *N* barium hydroxide containing 2% barium chloride. The total amount of barium carbonate is determined by titrating the excess barium hydroxide with 0.12 *N* hydrochloric acid using phenolphthalein as the indicator and calculating the amount of carbonate from the difference between the blank and the sample titrated. The barium carbonate precipitate was then collected and washed three times with 25 ml. portions of 95% ethanol. Several aliquots of each carbonate precipitate were homogenized in ethanol and plated on copper disks. Radioactivity measurements were made with a windowless flow counter (Tracerlab Windowless Flow Counter, Model SC-16) and an automatic scaling unit (Nuclear Scaling Unit, Model 163). These measurements were then corrected for background count (usually

20 ±2 counts/minute) and self-absorption (see Fig. 2) and calculated as specific activity (counts/minute/mg. of carbon).

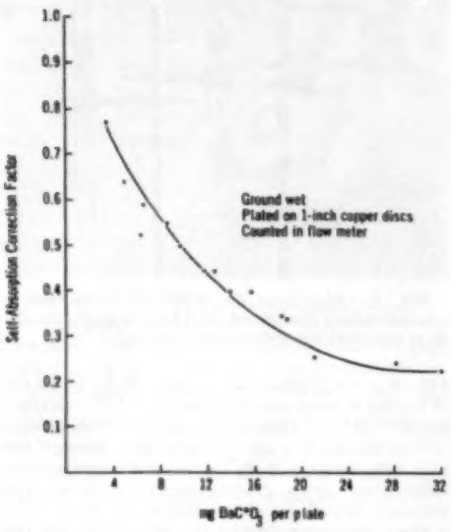

Fig. 2.—Self-absorption correction curve for BaC^*O_3

Samples of mycelium material were dried completely in a lyophilizer prior to oxidation. Samples of acetate or other radioactive liquids were neutralized with sodium hydroxide solution and evaporated to dryness under reduced pressure before oxidation. If the activity of a sample was too high, a smaller aliquot of the material was diluted with nonradioactive glucose and oxidized again in order to keep the counting rate between 100 and 2000 counts per minute.

Extraction and Isolation of Organic Acids—The organic acids were extracted by boiling 30 Gm. (wet weight) of mycelium pellets in 30 ml. of 0.1 *N* sodium hydroxide and filtering. The residue was re-treated in the same manner three times. The aqueous extracts were combined and neutralized, if necessary, with sodium hydroxide solution and evaporated to dryness. The dried extract was then taken up with 2.5 ml. of 0.2 *N* H_2SO_4 and chromatographed using the method of Eaton and Klein (12). At least four samples of each of the three groups of mycelial pellets described above were extracted and chromatographed. Each fraction was titrated with 0.01 *N* NaOH and the amount of acid in each fraction calculated. This information was plotted and the amount of each individual acid determined. The organic acids isolated from the acetate-treated, "starved" material were oxidized, their radioactivity counted, and specific activity calculated. The results are shown in Table I.

TABLE I.—COMPARISON OF ORGANIC ACID CONCENTRATION IN FRESH PELLETS, STARVED PELLETS AND ACETATE-TREATED PELLETS OF CLAVICEPS LITORALIS

	Mg Acid Isolated from Fresh Pellets	Mg Acid Isolated from Starved Pellets	Mg Acid Isolated Acetate-treated Starved Pellets[a]	Specific Activity[b]
Acetate	2.56	0.94	1.57	262
Pyruvate	..	2.75	7.28	335
Succinate	..	1.57	..	..
Fumarate	0.30	1.00	3.74	1307
Malate	0.32	1.43	3.28	2658
Total Acids	3.18	7.69	15.87	

[a] Carbon-14 carboxyl labeled sodium acetate.
[b] Specific activity = counts per minute per mg carbon.

DISCUSSION AND CONCLUSIONS

From the results obtained above it appeared that an oxidative cycle involving some of the components of the TCA cycle was in operation. A significant amount of acetate was isolated from the fresh "unstarved" material along with small amounts of fumarate and malate. The entire amount of acetate isolated should not be considered as coming from such an oxidative cycle; for, when a general extraction method is used, all acetate, whether "active" acetate or acetate from a metabolic "acetate pool," will be extracted.

The "starved" material showed approximately a 100 per cent increase in total acids isolated compared to the amount found in the fresh material, indicating perhaps that the conditions of aeration and starvation enhanced the oxidative processes and forced the organism to utilize reserve materials. The "starved," acetate-treated material showed a 200 per cent increase in total acids In both latter cases acetate was found in the least amount while pyruvate appeared in the greatest quantity. Fumarate and malate appeared in approximately the same amounts in each separate group of material. It is reasonable to believe that a portion of the carbohydrate would be oxidized through the cycle. With the cycle operating at a higher rate than in the fresh material, it could oxidize the labeled acetate at a more efficient pace.

In all cases no citrate or isocitrate could be isolated. This would indicate that the entire TCA cycle as elucidated by Krebs was not functioning, however, these acids may have been present in minute amounts too small to be detected or they may have been absent. The absence of citrate and isocitrate could also be explained on the basis of the work by Ochoa *et al.* (13, 14). These workers reported the finding of an enzyme present in microorganisms which not only decarboxylated *l*-malic acid, but also decarboxylated oxalacetic acid. The result of these decarboxylations was the formation of pyruvate which, in turn, was converted to lactic acid by lactic dehydrogenase. However, since lactic acid could not be detected in *C. litoralis*, the possibilty exists that lactic dehydrogenase was lacking or might have been inactive in the fungus allowing an accumulation of pyruvate to occur. Considering the above two points, it would seem reasonable to believe that the formation of citrate would be prevented to a great extent and such a situation would also explain the accumulation of pyruvate.

Although the TCA cycle could be prevented from operating in its entirety by such an enzyme, the cycle would exist in part if, through the Thunberg-Wieland reaction, the acetate were incorporated into the cycle in the form of succinate. Succinate was isolated from the "starved," untreated material, but the failure to isolate succinate from the "starved," acetate-treated mycelial pellets weakens such a hypothesis unless the presence of a highly active form of succinic dehydrogenase could be shown to exist in *C. litoralis*. No work along this line was attempted. From succinate the carbon could follow the pathway through fumarate and malate to pyruvate, and this acid could then be decarboxylated to form acetate.

From the results obtained it appears that the entire TCA cycle does not function in *C. litoralis* when it is grown under artificial conditions used in this study, but a partial cycle does exist as an oxidative pathway in this fungus. However, considering the low specific activity of the isolated, labeled acids, it is possible that some other oxidative pathway plays a more important role in the oxidative metabolism of *C. litoralis* than does any organic acid cycle which may resemble the complete TCA cycle.

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A Comparative Study of Surfactant Influence on the Release of Ions from an Emulsified Ointment Base*

By JOHN F. STARK†, JOHN E. CHRISTIAN, and H. GEORGE DeKAY

An apparatus has been designed and an *in vitro* method employing radioisotopes has been developed to study medicament release from pharmaceutical semisolids. Radiolabeled mercuric and iodide ions have been incorporated into hydrophilic bases emulsified with 1, 3, and 5 per cent concentrations of anionic, cationic, and nonionic surface active agents and the ability of the bases to release the contained ions has been measured

THE SUBSERVIENCE of the newer hydrophilic bases must of necessity be a measure of their ability to release a contained medicinal agent. The hydrophilic character of the emulsified semisolid, the surfactant classification, concentration and particle size, and the solubility and pH of the incorporated medicament appear, among other factors, to influence the release pattern.

An *in vitro* method, simulating the actual conditions under which a medicated ointment is frequently used, was developed to study medicament release. Because of the ease and extreme sensitivity of detection, radioisotope labeled compounds were employed to represent the medicinal fraction of the hydrophilic bases.

The release patterns of a modified U. S. P. XV hydrophilic ointment base (1), emulsified with various concentrations of ionic and nonionic surface active agents, have been measured over predetermined time intervals. The effect of surfactant classification, concentration and electrostatic influence on the ability of the emulsified vehicles to release the labeled ions has been studied.

EXPERIMENTAL

Design of the Apparatus.—The apparatus (Fig. 1) supported a section of $3^1/_4$ inch standard stretch seamless cellulose tubing,[1] securely attached to the apparatus in such a manner as to support a slight positive head of pressure and thus withstand the contact of the circulating liquid without leaking. The cellulose enabled more reproducibility of results than is usually possible with a living excised membrane.

The counting chamber was designed so as to allow fluid flow around the counter housing without actually wetting and contaminating the contained Geiger tube. An R.I.D.L. Model 200 Scaler[2] in conjunction with a bismuth cathode side-window

* Received July 24, 1957, from the Bio-Nucleonics Laboratory, School of Pharmacy, Purdue University, Lafayette, Ind.
Presented to the Scientific Section, A. PH. A., New York meeting, April–May, 1957.
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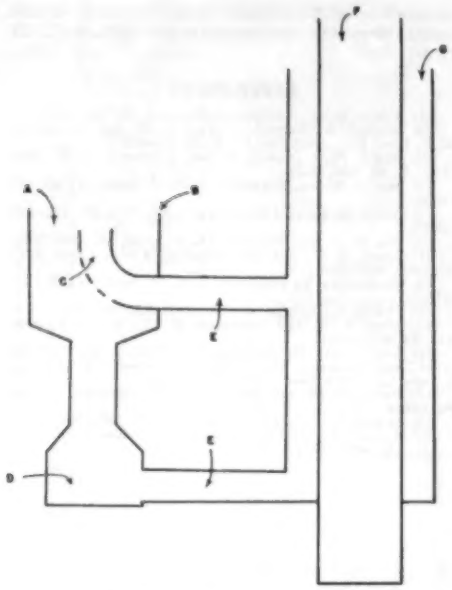

Fig. 1.—Diagram of the release apparatus. A, Membrane Station; B, Ground Glass Surface; C, Perforated Extension (to prevent an ionic concentration gradient below the membrane); D, Stirring Compartment; E, Connecting Arms; F, Counter Housing; G, Counting Chamber

counter,[3] geometrically seated in the counter housing, was used to determine the radioactivity. Shielding, in the form of lead shot contained in a copper housing designed to fit across the connecting arms, protected the counter from external sources of ionizing radiation.

A physiological solution containing the chlorides of sodium, potassium, calcium, and magnesium (2) was used as the circulating liquid. A rheostat controlled magnetic bar, enclosed in the stirring station, provided the necessary circulation.

A constant temperature bath of 2-liter capacity was constructed of sheet copper to accommodate the release apparatus. All experimental work was conducted at 37° ± 1°.

The bases containing the labeled ions were placed in a lucite holder and carefully weighed for application to the membrane. The holder was cut from 7-mm. stock, 50 x 65 mm. and a hole 32 mm. in diameter was bored in the center of the section. The lucite was backed with aluminum foil, forming a cup to support bases of uniform thickness, known weight, and total activity.

Base Preparation.—The U. S. P. XV hydrophilic ointment base was modified by using selected ionic and nonionic surfactants in 1, 3, and 5% concentrations as emulsifying agents. A constant aqueous phase was maintained by adjusting the amounts of white petrolatum and stearyl alcohol to accommodate the varying surfactant levels. A portion of the water was omitted in the formulation and subsequently replaced with dilutions of the radioactive solutions. With other than these changes, the U. S. P. method of formulation was followed.

The general formulas of the bases used are shown in Table I.

TABLE I

Ingredients	Quantities, Gm. Surfactant 1	Concn. 3	Per Cent 5
Methylparaben	.025	.025	.025
Propylparaben	.015	.015	.015
Stearyl alcohol	27.0	26.0	25.0
White petrolatum	27.0	26.0	25.0
Emulsifier	1.0	3.0	5.0
Propylene glycol	12.0	12.0	12.0
Purified water	23.0	23.0	23.0
Labeled dilution[a]	10.0	10.0	10.0
To make about	100.0	100.0	100.0

[a] Refers to either NaI^{131} or $Hg^{203}(NO_3)_2$ dilutions containing 50 mg./ml. of carrier.

Sodium radio-iodide (I^{131}) and mercury (Hg^{203}) nitrate,[4] containing a labeled anion and cation respectively, were employed to simulate the medicament fractions of the bases. These isotopes have convenient halflives and emit characteristic gamma radiations of nearly the same energy. Dilutions containing 50 mg./ml. of carrier were prepared so that the volume incorporated imparted 1,500 to 5,000 counts per minute[5] as determined by the procedure described below.

A portion of the base containing the labeled medicament was placed in a 2-dram, collapsible, tin tube for convenience in filling the lucite holder and weighing of the standardization sample.

Base Standardization.—An accurately weighed representative sample of radioactive ointment of approximately 0.5 Gm. was dispensed from the collapsible tube into a 100-ml. volumetric flask. The base was dissolved in a warm mixture of equal parts of ethyl alcohol and chloroform, the solution cooled, and brought to volume with the solvent mixture.

The 100 ml. of solution was added to a standardization apparatus modeled after and identical to the counting section of the release apparatus and the radioactivity determined. The activity was expressed in terms of CPM per unit weight of base and was used to calculate the total activity of the base applied to the membrane in the release study.

The standardization of the base under conditions identical to those in the determination of the release activity enabled expression of the final results in terms of per cent of the total medicament applied to the membrane.

Medicament Release Studies.—The magnetic bar was placed into the stirring compartment and the membrane attached to the release apparatus. The physiological solution was added and the air pocket beneath the membrane was removed by displacement. The apparatus was stationed in the 37° bath, the shielding was placed in position, and the counter was inserted into the housing. Rheostat controlled stirring was commenced and an additional physiological solution was added up to a

[3] Nuclear-Chicago, Chicago, Ill.

[4] Obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn. Operated for the United States Atomic Energy Commission by Union Carbide Nuclear Company.

[5] Counts per minute, hereinafter referred to as CPM.

predetermined and constant height on the counter housing. This insured that the liquid level with regard to the pre-positioned counter was identical to that used in the standardization procedure and subsequent determinations.

Background activity determinations were made in each instance with the lucite holder (foil side down) in position on the membrane; thus, any small fraction of the ionizing radiation which penetrated the shielding was recorded as part of the background. The lucite holder was then inverted and the foil backing of the holder was rubbed gently to promote uniform contact between the base and the membrane.

A stopwatch was started immediately after applying the base to the membrane and after four minutes had elapsed, the radioactivity of the physiological solution was determined over a period of two minutes. The corrected activity, expressed in CPM, was divided by the total activity of the base applied to the membrane, and multiplied by 100. This relative value, expressed as per cent, was used as a measure of medicament release at the five-minute interval. Similar activity measurements indicated the release at the end of 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120, 135, 150, and 170 minutes.

The experimental data for iodide and mercuric ion are presented in Table II. Although the release measurements were conducted at sixteen different time intervals, only values indicative of the release at the end of two hours are reported for conciseness.

DISCUSSION

A survey of the data revealed that the ability of the hydrophilic bases to release the ions under study followed a characteristic pattern, especially with regard to surfactant concentration and type.

Maximum release of both the iodide ion and mercuric ion was evidenced from bases emulsified with 1% surfactant concentrations. The bases emulsified with 3% concentrations exhibited a decreased ability to release the incorporated ions. At the 5% level the release was generally further retarded. In an earlier publication Barker, *et al.* (3), reported that iodide was released to a greater extent from hydrophilic bases emulsified with low rather than higher levels of nonionic surfactants, as measured by a modified agar plate method. The results obtained by the method reported here are in agreement with Barker's findings. Maximum release within each group, ionic or nonionic, was generally noted at the lowest concentration level employed, namely 1%.

The ionic or nonionic nature of the agents utilized to emulsify the vehicles substantially influenced the release. The nonionic group of surfactant promoted the release of both the iodide and mercuric ions to a greater extent than did the anionic or cationic group. In the case of iodide ion, the anionic surfactants provided for greater release than the cationic; whereas, in the case of the mercuric ion there was no significant difference between the effects of these two types.

TABLE II.—THE RELEASE OF IODIDE AND MERCURIC IONS[a] FROM HYDROPHILIC OINTMENT BASES[b] EMULSIFIED WITH 1, 3, AND 5 PER CENT CONCENTRATIONS OF IONIC AND NONIONIC SURFACE ACTIVE AGENTS

Surfactant Listing[c]	Iodide Ion Released[d], Concn. of Surfactant, %			Mercuric Ion Released[d], Concn. of Surfactant, %		
	1	3	5	1	3	5
Aerosol® OT	18.5	13.2	9.8	8.1	4.6	3.5
Antarate K	14.5	9.1	10.9	5.7	2.3	1.7
Blendene	20.4	14.7	11.3	5.0	3.9	3.2
Emulgade F	18.1	16.2	16.6	7.7	5.6	3.8
Sodium lauryl sulf.	17.4	16.3	14.8	6.3	5.9	6.1
Soluble base 11	15.6	12.2	9.8	5.9	2.1	2.5
BTC 100%	7.9	7.8	6.7	3.2	1.1	1.0
Emcol E-607S	12.0	8.5	6.4	6.7	2.4	2.0
Ethomeen C/15	16.7	19.4	17.3	6.7	2.6	2.8
Ethyl cetab	11.4	8.0	8.0	5.7	1.6	1.6
Katapol PN-430	14.7	8.6	7.0	4.8	2.4	2.1
Triethanolamine	11.3	10.4	8.9	6.2	5.0	2.9
Brij® 35	21.8	15.4	16.2	10.3	5.1	3.7
Emulsifier L-32	20.0	4.7	4.6	6.2	4.0	2.4
Ethomid C/15	13.7	12.5	10.9	7.5	4.7	3.3
G-2000C	21.0	11.9	9.9	11.5	7.0	4.3
G-2152	22.9	19.1	16.4	10.7	7.1	5.9
G-7596J	23.9	17.2	18.3	11.4	6.5	5.6
Myrj® 52	21.1	17.5	17.2	9.9	8.9	6.1
Neutronyl 834	20.9	7.7	4.4	7.5	3.0	3.7
Nonisol® 100	20.2	16.9	15.8	10.6	6.0	5.5
Promulgen	22.7	17.9	14.8	13.0	7.9	6.4
Tween® 40	26.1	17.6	19.0	12.4	6.6	4.9
Tween 80	26.3	20.3	20.4	8.8	6.4	2.5

[a] The iodine and mercury were in the chemical forms NaI and $Hg(NO_3)_2$ respectively.

[b] The hydrophilic bases were modifications of the U. S. P. XV hydrophilic ointment. Ten per cent water was omitted in formulating the bases and subsequently replaced with the labeled dilutions. The aqueous phase of each base was constant and the increased amounts of emulsifier were compensated for by equally reducing the amounts of stearyl alcohol and white petrolatum.

[c] Surfactants 1–6 are classified anionic; 7–12 are classified cationic; and 13–24 are classified nonionic. J. W. McCutcheon's "Synthetic Detergents and Emulsifiers—Up To Date," III, 1955, may be consulted for suppliers of the surface-active agents employed in this study.

[d] The values reported represent the medicament released at the end of 120 minutes. A study of the variation that could be expected between individual determinations was conducted and percentage errors of 2.3 to 4.1 were found, well within the expected deviation.

CONCLUSION

It can be generally stated that maximum release of a medicinal agent may be expected from a base emulsified with a nonionic surfactant in as low a concentration as is feasible to impart the required emulsion character and stability; however, the release of a given medicament is dependent upon many factors and each formulation must be considered individually for medicament release characteristics.

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The Paper Chromatography and Electrophoresis of Selected Therapeutic Agents I.*

The Paper Chromatography of Antihistaminic Agents

By BETLEIGH C. COX, HAROLD C. HEIM, and CHARLES F. POE

The paper chromatography of some antihistamines has been performed with six solvent systems. Pretreatment of the paper with monosodium citrate resulted in consistently lower and more widely varying R_f values, in all of the solvent systems used. A greater degree of resolution was obtained. The solvent systems that provided the most successful resolution of a mixture of the antihistamines were: (*a*) isoamyl alcohol-water-citric acid, 50:50:3 Gm.; (*b*) *n*-butanol-water-amyl acetate, 10:1:4 v/v; and (*c*) isoamyl alcohol-water-ethyl acetate, 10:1:3 v/v.

THERE ARE NUMEROUS antihistaminic compounds in use today, and many exhibit similarities of chemical structure. The problem of identification and resolution of these substances has become complex. The antihistamines have been analyzed by means of their melting points, spectrophotometric patterns (1, 2), polarographic curves (3), qualitative reactions (4, 5), and ion-exchange resin behavior (6).

Paper chromatography offers a method of identification and recovery of minute amounts of these compounds. The resolution of a mixture of the antihistamines is made possible by the use of this technique. This paper describes the paper chromatographic behavior of a representative group of the antihistamines.

EXPERIMENTAL

Apparatus.—The chromatographic chamber was a Research Equipment Corp. Chromatocab, Model A 300.

Materials.—All solvents and chemicals were C.P. or Analytical Reagent grade.

The following compounds were studied: Diphenhydramine hydrochloride (Benadryl®), thonzylamine hydrochloride (Neo-hetramine®), doxylamine succinate (Decapryn®), tripelennamine hydrochloride (Pyribenzamine®), chlorcyclizine hydrochloride (Di-Paralene®), pyrilamine maleate (Neo-Antergan®), pyrathiazine hydrochloride (Pyrrolrzote®), antazoline hydrochloride (Antistine®), methapyrilene hydrochloride (Thenylene®), pheniramine maleate (Trimeton®), chlorpheniramine maleate (Chlor-Trimeton®) and phenindamine tartrate (Thephorin®).

Solvent Systems.—(*a*) *n*-Butanol-water-citric acid, 50:50:3 Gm.; (*b*) isoamyl alcohol-water-citric acid, 50:50:3; (*c*) *n*-butanol-water-ethyl acetate, 10:2:1 v/v; (*d*) *n*-butanol-water-ethyl acetate, 10:1:2 v/v; (*e*) isoamyl alcohol-water-ethyl acetate, 10:1:3 v/v; (*f*) *n*-butanol-water-amyl acetate, 10:1:4 v/v.

The mobile phase was the organic layer, in all instances. In all experiments involving *n*-butanol, fresh solvent was necessary for each resolution to achieve reproducible R_f values.

Paper.—Whatman No. 1 chromatography paper, in strips 34.0 cm. long and 1.3 cm. wide, was used. Pretreated strips were used in each solvent system. Pretreatment consisted of thoroughly wetting the paper in 5 per cent aqueous monosodium citrate, blotting the excess solution and drying at 70° for 20 minutes.

Experimental Procedure.—Descending chromatography was used. A solution of approximately 25 mcg. of antihistamine in 95% ethanol was applied to each strip. After drying, the strips were placed in the Chromatocab for an equilibration period of

* Received July 18, 1956, from the College of Pharmacy, University of Colorado, Boulder.
Presented to the Scientific Section, A.Ph.A., Detroit meeting April 1956.

nine hours. A constant volume of 150 ml. of developing solvent was added to the trough in all experiments. Seventy-five ml. of the aqueous phase were placed in the bottom of the chamber. Development was performed at 23° ± 0.5°. The strips were air dried and sprayed with modified Dragendorff reagent (7) for detection of the compounds.

RESULTS AND DISCUSSION

The R_f values of the antihistamines, as determined in the various solvent systems, are listed in Table I. Although 18 determinations were made with each antihistamine, only the most characteristic ones are included in the table. Also, the results with untreated paper are omitted. Some experiments were performed in which the ascending method was used.

With the apparatus used in this study, the R_f values were found to be essentially the same as those obtained with the descending method.

The use of a large chromatographic chamber necessitated an adequate equilibration period and a strict control of quantities of mobile and stationary solvent phases. The development period of nine hours produced a solvent flow of 20.0 to 22.0 cm., and increased accuracy. R_f values were slightly erratic if the solvent flow was permitted to reach a point two or three cm. from the end of the strip.

Pretreatment of the paper with monosodium citrate produced a significant improvement in the chromatography of these compounds. The spots were more discrete and more readily visible. The background was initially colorless, whereas upon untreated strips, the background was initially orange. The resolution of a mixture of compounds was improved, in most instances.

The addition of acetic acid as the third component to a *n*-butanol-water solvent system resulted in high R_f values, and poor resolution. Lactic acid as the third component produced no improvement. The solvent system *n*-butanol-water-citric acid, 50:50:1 Gm. gave lower R_f values, but unsatisfactory resolution, upon untreated strips. However, pretreated strips produced considerably improved resolution of the compounds. Increasing the citric acid content to 3 Gm. further improved the resolution. When the citric acid content was increased to 5 Gm., the resolution was decreased because there were a group of R_f values in a high range, and a group in a low range. Thus, it appears that optimum resolution of these compounds is attained with the solvent system *n*-butanol-water-citric acid 50:50:3.

When isoamyl alcohol was used as the solvent, the R_f values were consistently lower than when *n*-butanol was used. Some compounds, such as chlorcyclizine hydrochloride, pyrathiazine hydrochloride, and phenindamine tartrate, exhibited high R_f values regardless of the solvent system used. On the other hand, compounds such as doxylamine succinate, pyrilamine maleate, and pheniramine maleate exhibited generally lower R_f values and the magnitude of R_f was found to be dependent to a considerable extent upon the solvent system used. The R_f values of pyrathiazine hydrochloride varied between 0.74 and 0.96; those of methapyrilene hydrochloride between 0.19 and 0.73. This characteristic is useful as a guide to the selection of a resolving system.

TABLE I.—RF VALUES OF ANTIHISTAMINES

Compound	Solvent System[a] 1	2	3	4	5	6
Diphenhydramine hydrochloride	0.87	0.75	0.94	0.68	0.72	0.65
Thonzylamine hydrochloride	0.79	0.56	0.88	0.56	0.45	0.49
Doxylamine succinate	0.39	0.16	0.59	0.38	0.20	0.28
Tripelennamine hydrochloride	0.49	0.23	0.70	0.42	0.28	0.31
Chlorcyclizine hydrochloride	0.97	0.79	0.97	0.80	0.77	0.69
Pyrilamine maleate	0.48	0.31	0.68	0.39	0.26	0.33
Pyrathiazine hydrochloride	0.90	0.88	0.96	0.79	0.74	0.76
Antazoline hydrochloride	0.88	0.84	0.95	0.78	0.67	0.63
Metapyrilene hydrochloride	0.50	0.19	0.73	0.44	0.29	0.35
Pheniramine maleate	0.44	0.25	0.61	0.40	0.23	0.24
Chlorpheniramine maleate	0.78	0.49	0.86	0.49	0.35	0.43
Phenindamine tartrate	0.89	0.81	0.93	0.73	0.70	0.72

[a] 1. *n*-Butanol-water-citric acid, 50:50:3 Gm., with pretreated paper. 2. Isoamyl alcohol-water-citric acid, 50:50:3 Gm., with pretreated paper. 3. *n*-Butanol-water-ethyl acetate, 10:2:1 v/v, with pretreated paper. 4. *n*-Butanol-water ethyl acetate, 10:1:2 v/v, with pretreated paper. 5. Isoamyl alcohol-water-ethyl acetate, 10:1:3 v/v, with pretreated paper. 6. *n*-Butanol-water-amyl acetate, 10:1:4 v/v, with pretreated paper.

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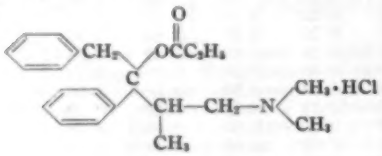
Notes

A Note on the Crystallography of *d*-Propoxyphene Hydrochloride*

By HARRY A. ROSE

A NEW COMPOUND, *d*-propoxyphene hydrochloride, prepared at the Lilly Laboratories has about the same analgesic response as codeine phosphate (1). The chemistry of the compound is discussed elsewhere (2, 3). Data are given here which permit the identification of this compound by crystallographic methods.

Chemically, *d*-propoxyphene hydrochloride is α-*d*-4-dimethylamino-1,2-diphenyl-3-methyl-2-propionyloxybutane hydrochloride and has the following structure:



EXPERIMENTAL

Crystallization from ethyl acetate resulted in orthorhombic prisms elongated parallel to the *b* axis. The crystals showed the prism {110} and the orthodome {101}. Measurements of the interfacial angles gave the following results: $110 > \bar{1}10$ (polar) 94° 18′ (optical), 93° 58′ (X-ray); $101 > \bar{1}01$ (polar) 86° 03′ (optical), 86° 10′ (X-ray).

The X-ray powder diffraction data were obtained using copper radiation and nickel filter with a camera 114.6 mm. in diameter. A wavelength value of 1.5405 *A* was used in the calculations. The indexing of the powder pattern was done on the basis of single crystal rotation patterns around both the *b* and *c* axes.

On heating, *d*-propoxyphene hydrochloride melts in the range 168–171°. The melt does not crystallize on cooling.

TABLE I.—X-RAY CRYSTALLOGRAPHIC DATA

Unit Cell Dimensions.	$a_0 = 12.83\ A$, $b_0 = 13.75\ A$, $c_0 = 12.00$
Formula Weight per Cell.	4.
Formula Weight.	375.9.
Density.	1.173 Gm./cc. (flotation); 1.181 Gm./cc. (x-ray).
Axial Ratio.	$a:b:c = 0.9331:1:0.8727$
Space Group.	$P2_12_12_1$

TABLE II.—OPTICAL CRYSTALLOGRAPHIC DATA

Refractive Indices.	(5893 *A*, 25°). $\alpha = 1.560$ $\beta = 1.582$, $\gamma = 1.638$
Optic Axial Angle.	(+) $2V = 66°$ (calcd. from α, β and γ).
Optic Axial Plane.	100
Acute Bisectrix.	$\gamma = c$

TABLE III.—X-RAY POWDER DIFFRACTION DATA

d	I/I_1	*h k l*	*d* (*calcd.*)
9.50	0.03	110	9.38
8.79	0.33	101	8.76
7.40	0.20	111	7.39
6.39	0.03	200	6.41
6.02	1.00	120, 002, 021	6.06, 6.00, 5.97
5.60	0.03	201	5.36
5.38	0.03	121	5.41
5.06	0.27	112	5.05
4.55	0.20	022	4.52
4.38	0.20	202, 221	4.38, 4.37
4.08	0.67b	212, 310, 131, 301	4.17, 4.08, 4.06, 4.03
3.86	0.07	311	3.87
3.76	0.20	230	3.73
3.65	0.07	320	3.63
3.50	0.20	132, 302, 321	3.50, 3.48, 3.47
3.35	0.07	123	3.34
3.21	0.13	400, 141	3.21, 3.20
3.10	0.07	401	3.10
3.01	0.07	240, 331, 411	3.03, 3.03, 3.01
2.92	0.13	303, 142	2.92, 2.91
2.86	0.07	313	2.86
2.78	0.03	332	2.77
2.70	0.03	204	2.72
2.62	0.07	151, 341	2.62, 2.61
2.52	0.13	224, 510	2.53, 2.52
2.46	0.07		
2.40	0.03		
2.34	0.07b		
2.27	0.03		
2.23	0.03		
2.14	0.03b		
2.09	0.03b		
2.02	0.03		
2.00	0.03		
1.95	0.03		
1.89	0.03		

* Received July 1, 1957 from the Analytical Department, Eli Lilly and Company, Indianapolis, Ind.

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A Note on Specific Rotation and Temperature Coefficient*

By JOSEPH ROSIN and C. J. WILLIAMS

IT IS UNIVERSALLY accepted that the temperature at which it is determined affects optical rotation as it does other physico-chemical properties, e. g., specific gravity, refractive index, etc. For this reason numerical data of these properties are either "suffixed" with the temperature at which the data apply or were measured, or it is implied by specific statements in the introductory remarks. In the United States Pharmacopœia and the National Formulary the temperature for measuring rotation is 25°; in the British Pharmacopœia and the majority of European Pharmacopoeias it is 20°.

In the past several years the senior author has had correspondence with several laboratories about reported divergences in the specific rotation between laboratories on the same samples. This situation came to focus in a collaborative study of the purity of an amino acid. Discrepancies in the reports on the specific rotation were of a puzzling magnitude. Normal variations in concentration, in the authors' experience even as much as 50%, would not account for the differences. Nor could they be attributed, so it was thought, to normal variations in laboratory temperatures. In chemical laboratories it is generally assumed that a deviation of ± 2–3° from the standard temperature has no significant effect on specific rotation value. As a matter of last resort we decided to check experimentally the effect of temperature variations within approximately the range of usual laboratory temperatures.

The solution of the chemical in a 100 mm. polarimeter tube fitted with a thermometer was cooled to the low temperature (20° or 22°) and the rotation measured by taking the mean of 6 to 10 readings. The temperature of the solution was then raised by warming one or two degrees and the rotation determined as before described. After observing the rotation at the highest temperature (30°) the solution was cooled to the original lowest temperature and the rotation again determined at this temperature. This was done to make sure that no deterioration of the chemical had occurred during the intervals. The first value found at the lowest temperature and that of the check did not deviate from each other by at most one degree of specific rotation.

The temperature "coefficient"—the increase or decrease in specific rotation per degree difference in temperature—was thus found surprisingly large for the chemical first investigated, and when the reported values by the several laboratories were corrected for the temperature at which they were determined by the coefficient we established, the results were harmonious.

This finding induced us to test several other medicinal chemicals for their specific rotation temperature coefficient. See the results in Table I.

The specific rotation values in the Table are on the anhydrous basis. The solvent, when not otherwise indicated in the last column, is water.

Of the 16 specimens thus examined, 6 showed a not insignificant temperature coefficient, and half of them a rather large one. This study emphasizes what is well known in science that assumptions are not to be relied upon.

TABLE I.—SPECIFIC ROTATION–TEMPERATURE COEFFICIENTS OF CERTAIN MEDICINAL CHEMICALS

	Temperatures at which Determined													
	20°	21°	22°	23°	24°	25°	26°	27°	28°	29°	30°	Difference[a]	Temp. Coeff.	Concn.
L-Arginine HCl	+21.8°	...	...	21.6°	...	21.4°	...	21.4°	...	...	21.2°	0.6°	0.06°	C = 5[b]
Ascorbic Acid	....	....	+21.4°	...	21.3°	21.3°	21.2°	...	21.3°	21.1°	21.1°	0.3°	0.03°	C = 10
Cinchonine	....	....	+230°	...	229°	...	228°	...	228°	...	227°	3.0°	0.33°	C = 1[c]
Codeine Sulfate	−113.4°	...	113.4°	113.2°	...	113.2°	...	112.9°	...	112.9°	112.9°	0.5°	0.05°	C = 2
Colchicine	−439.7°	436.1°	432.4°	428.3°	422.0°	421.2°	418.3°	414.7°	412.6°	410.4°	409.0°	30.7°	2.8°	C = 1
L-Cystine	−213.9°	212.0°	210.7°	209.5°	208.7°	206.7°	204.5°	202.7°	200.4°	199.3°	...	14.6°	1.46°	C = 3.5[b]
Dehydrochloric Acid	....	...	+31.0°	31.0°	30.8°	...	31.3°	31.0°	...	31.0°	31.0°	0.0°	0.0°	C = 2[d]
Dextrose	....	...	+53.0°	52.6°	...	52.8°	52.6°	52.6°	52.8°	...	52.6°	0.4°	0.04°	C = 5
Dihydrostreptomycin SO_4	....	−86.8°	86.6°	86.4°	86.2°	86.2°	86.2°	...	85.8°	85.8°	85.6°	1.2°	0.12°	C = 5
Ephedrine Hydrochloride	....	−34.2°	34.2°	34.2°	34.2°	34.0°	34.0°	34.2°	34.0°	34.0°	33.8°	0.4°	0.04°	C = 5
Ephedrine Sulfate	....	−31.2°	31.0°	31.2°	31.0°	31.0°	31.0°	31.0°	31.0°	31.0°	31.0°	0.2°	0.02°	C = 5
Hydrocortisone Acetate	+168.3°	167.8°	167.3°	166.5°	165.8°	165.8°	164.8°	...	164.5°	...	164.5°	3.8°	0.34°	C = 1.5[d]
L-Methionine	−7.9°	...	7.8°	...	7.6°	...	7.5°	...	7.2°	...	7.1°	0.8°	0.07°	C = 4
Penicillin Sodium	+296.8°	294.9°	293.4°	292.1°	291.1°	290.8°	289.5°	287.8°	287.8°	287.3°	286.9°	9.9°	0.9°	C = 3
Pilocarpin Nitrate	....	...	+82.3°	82.2°	81.7°	81.0°	80.7°	80.2°	80.0°	79.7°	79.5°	2.8°	0.3°	C = 3
Strychnine Sulfate	....	....	−27.8°	27.5°	27.1°	27.1°	27.1°	26.6°	26.6°	26.5°	26.2°	1.6°	0.18°	C = 7.5

[a] Difference in degrees of specific rotation determined at highest and lowest temperatures. [b] In 1 *N* HCl. [c] In ETOH + $CHCl_3$. [d] In Dioxane.

* Received November 15, 1937 from the Laboratories of Merck & Co., Rahway, N. J.

Book Notices

La Rauwolfia. By LIVIO MEDIANI. Casa Editrice Ambrosiana, Milan, 1957. xvi + 582 pp. 15.5 x 22 cm.

This book reviews, in a competent manner, the history, chemistry, and pharmacology of the several known species of Rauwolfia. It includes references to the original literature, but has no index.

Science looks at Smoking: A New Inquiry into the Effects of Smoking on Your Health. By ERIC NORTHRUP. Coward-McCann, Inc., New York, 1957. 190 pp. 13.5 x 20.5 cm. Price $3.

An effort is made to inquire into the effects of smoking, and to appraise some of the reports that have been so widely circulated. The reports on smoking as a cause of lung cancer and heart failure are reviewed and evaluated. The author points out that faulty research methods and preconceived attitudes have made some of the publicized conclusions invalid and suspect, and that as a result the public has been unduly alarmed by the hasty release of inconclusive reports and findings.

Tables for Use with Binomial Samples. By DONALD MAINLAND, LEE HERRERA, and MARION I. SUTCLIFFE. Department of Medical Statistics, New York University, New York, 1957. xix + 83 pp. 27.5 x 21 cm. Price $2.

The statistical tables published by Mainland and his collaborators during the past eight years are of pharmaceutical interest. All of the tables, with instructions, are now reissued in book form by photo-offset from typed material, with a stiff (twillpex) cover and spiral binding. It contains many more entries than in the original versions, in order to minimize the need for interpolation. The tables remove the need for computation or reduce it to a line or two of simple arithmetic. Tables I–IV are for the significance testing of fourfold contingency tables (comparison of two percentage frequencies). Tables V–IX show binomial confidence limits for sample sizes from 1 to 100,000, with intervals of 1% or less between sample percentages for the 95% and 99% limits, and somewhat wider intervals for the 80% limits. Estimates of sample sizes required under various conditions are obtainable from all the tables, but Table X is specially designed for this.

Veterinary Toxicology (Formerly Lander's Veterinary Toxicology). By R. J. GARNER. The Williams & Wilkins Company, Baltimore, 1957 (distributors for Baillière, Tindall and Cox, London). iv + 415 pp. 15 x 23 cm. Price $7.50.

This book was published originally under the title "Lander's Veterinary Toxicology," the last edition of which appeared in 1945. Since that time, so much new material has accumulated that it has been necessary to prepare a complete revision. Most toxic compounds to which animals may be exposed have been covered by dividing them into six classifications. These classifications include inorganic substances; organic compounds: (I) drugs; (II) pesticides; and (III) miscellaneous. In two other chapters poisonous plants and radioactive materials are discussed from a toxicological standpoint. The eighth and final chapter covers quite adequately methods employed in toxicological analysis.

Volumetric Analysis, Vol. III-Titration Methods: Oxidation-Reduction Reactions. By I. M. Kolthoff and R. Belcher with the cooperation of V. A. Stenger and G. Matsuyama. Interscience Publishers, Inc., New York, 1957. ix + 714 pp. 15 x 23 cm. Price $15.

The third and last volume of "Volumetric Analysis" deals exclusively with oxidation-reduction titrations. It is logically arranged in a series of fifteen chapters, is well documented throughout, and is provided with author and subject indexes. The first chapter discusses reactions, indicators, and general techniques in oxidation-reduction titrations. Other chapters discuss potassium permanganate as a volumetric reagent, oxidimetry with permanganate and with ceric salts, potassium dichromate as oxidizing titrant, iodometry, determination of water with the Karl Fischer reagent. Other chapters cover the use of potassium iodate as an oxidizing titrant; oxidation of organic compounds with periodate; potassium bromate as a titrimetric reagent; oxidation with hypohalites; titrations with iron, titanium, and other strong reducing agents; and miscellaneous titrants. The book constitutes a useful reference to titration methods involving oxidation-reduction reactions.

Advances in Pest Control Research. Vol. I. Edited by R. L. METCALF. Interscience Publishers, Inc., New York, 1957. vii + 514 pp. 15 x 23 cm. Price $11.

This is the first in a series of selected contributions by specialists in fields related to pest control research. The pests include economically destructive species of plants and animals, variously classified as: viruses, bacteria, fungi, weeds, protozoa, nematodes and other helminths, molluscs, insects and other arthropods, fish, birds, and rodents. The text of this volume includes the following subjects: Control of health hazards associated with the use of pesticides, The chemistry and mode of action of herbicides, Uses of radioisotopes in pesticide research, The chemistry of action of organic phosphorus insecticides, Mechanisms of fungitoxicity, Recent advances in control of soil fungi, Repellents for biting arthropods, The status of systemic insecticides in pest control practices, Chemical analysis of pesticide residues, and Bioassay of pesticide residues. The text includes many references which are given at the end of each chapter. A good subject index is appended.

The style, format, type, and binding of the book are very good. The text material represents good, comprehensive reviews with suggested approaches to

further research. The book should be very useful in public health, pharmacy, chemistry, agriculture, and analytical libraries.

Basic Facts of Pharmacology. By S. M. BROOKS. W. B. Saunders Co., Philadelphia, Pa., 1957. 323 pp. 14 x 20.5 cm. Price $4.

This textbook is designed to present the essential facts of pharmacology to the members of the nursing profession—especially student nurses. The author claims little originality for most of the text except the organization and literary style. The latter are excellent. Included is a tabulation of important drugs in current use—giving generic name, common or trade name, U.S.P., N.F., or N.N.R. listing, and classification, use or condition treated. A glossary and a general index are appended.

Basic Pharmacology for Nurses. By JESSIE E. SQUIRE. The C. V. Mosby Co., St. Louis, 1957. 265 pp. 17.5 x 25.5 cm.

This book is a good text for teaching the required pharmacology to nurses. The arrangement of material is intended to aid in memorizing the information, and the "assignments" include good review questions. A glossary of frequently used terms and an index are included.

Cosmetics: Science and Technology. Edited by EDWARD SAGARIN. Interscience Publishers, Inc., New York, 1957. xix + 1,433 pp. 16.5 x 24.5 cm. Price $25.

This comprehensive compilation of technical information on cosmetics and practical formulations is a very useful addition to the literature in its field. The text is divided into four major divisions headed: The scope of cosmetics, Toilet preparations (about 800 pages), Manufacture and technology, Physiological considerations, and Legal considerations.

The similarity between cosmetic formulations and pharmaceutical lotions, ointments, aerosols, etc., make this book a very valuable reference for the pharmacy as well as the cosmetic laboratory. The text is documented with references that are given at the end of each chapter. An index of trade names of materials used in cosmetic formulations, a list of suppliers, an author index, and a subject index are appended.

The type, format, printing, and binding are very good in this book that should find a place in many laboratories and libraries.

The Dermatologist's Handbook. By ASHTON L. WELSH. Charles C Thomas, Publisher, Springfield, Ill., 1957. xii + 427 pp. 21.5 x 28 cm. Price $15.

This book represents an attempt to organize into a rational systematic classification, for purposes of instruction and reference, the topical and internal therapeutic agents concerning which a dermatologist must have knowledge. It is noted that synthetics, detergents, excitants, irritants are with us and about us; and that the dermatologist is confronted daily with manifestations of sensitivity to external agents and to internal agents consumed voluntarily or prescribed by other physicians. The book is an arrangement utilizing pharmaceutical dosage forms and pharmacologic classifications for external preparations and therapeutic indications for grouping preparations used internally.

Although the compilation is not intended to cover all classes of drugs completely, there are enough "representative compositions" to give the impression that the title "Dermatologist's Formulary" might be more suitable for this volume. The information, which is presented in abstract monograph form, should be useful as a ready reference. The book is very well printed, has a good format and clear type, and has a good binding. An index of manufacturers and distributors and a subject index are appended.

Hormonal Regulation of Energy Metabolism. Compiled and edited by LAURANCE W. KINSELL. Charles C Thomas, Publisher, Springfield, Ill., 1957. xii + 242 pp. 15 x 23 cm. Price $5.25

This book records the papers and discussions of a Conference on Hormonal Regulation of Energy Metabolism. Subjects included are: Certain aspects of hormonal regulation of carbohydrate metabolism, Hormonal regulation of enzymatic activity, The anterior pituitary in relation to energy metabolism, The mechanism of the influence of pituitary growth hormone on metabolism, The thyroid in relation to energy metabolism, Insulin—reminiscences, Diabetes and the insulin problem, and The adrenal cortex and energy metabolism. The importance of insulin in overall energy metabolism is stressed by its frequent inclusion in discussions of all phases of the problem.

The book has a good style, with excellent type, format, and binding. Its one shortcoming is the absence of a general index, which detracts from its usefulness as a reference volume.

Rogers' Inorganic Pharmaceutical Chemistry. 6th ed. By TAITO O. SOINE and CHARLES O. WILSON. Lea & Febiger, Philadelphia, 1957. 705 pp. 15.5 x 23.5 cm. Price $9.50

The textbook, that was originally written by Charles H. Rogers, has again been revised to be used readily with U. S. P. XV and N. F. X by students and practitioners of pharmacy. Aside from the modernization of the text material, changes from the 5th ed., which was reviewed in THIS JOURNAL, 41, 339(1952), include: deletion of discussions of nonofficial compounds of interest only from a chemical standpoint; deletion of discussions of pharmaceutical preparations in which official inorganic compounds occur, and provision of a statement on the purpose of these compounds in each preparation. The text follows closely the Periodic Table arrangement of the elements.

The text, type, format, and binding are very good. The inclusion of the kind of information that has been useful and helpful in the past assures the continued popularity of this book.

Pharmacology and Oral Therapeutics. 11th ed. By EDWARD G. DOBBS. The C. V. Mosby Company, St. Louis, 1957. 579 pp. 14 x 22 cm. Price $9.

This is the 11th edition of a well-known textbook

formerly published under the title "Pharmacology and Dental Therapeutics." It now contains new material pertinent to the field of oral medicine. It covers many new drugs that have appeared since 1951 and the descriptive material conforms to the current revisions of the United States Pharmacopeia and the National Formulary.

Chemical Properties of Organic Compounds, an Introduction. By ELLIOT N. MARVELL and ALBERT V. LOGAN. John Wiley & Sons, Inc., New York, 1957. x + 326 pp. 14.5 x 23 cm. Price $4.75.

This book uses theoretical organic chemistry as a basis for teaching introductory organic chemistry. Reactions have been chosen to emphasize principles and reduce the reader's memory burden. The concept of studying "functional groups" is stressed, and special topics are limited to those involving chemistry that will be useful to those this book was designed to serve.

Textbook of Pharmacognosy. 7th ed. By GEORGE EDWARD TREASE. The Williams and Wilkins Company, Baltimore, 1957. viii + 806 pp. 13.5 x 21.5 cm. Price $8.50.

The 7th edition of this standard textbook by Professor Trease has been thoroughly revised and includes more information than heretofore on the newer physical and chemical methods of drug evaluation. The botanical side of pharmacognosy, however, has not been deemphasized.

Aids to Materia Medica and Therapeutics. 5th ed. By J. W. HADGRAFT. Bailliere, Tindall and Cox, London, 1957, (The Williams and Wilkins Company, Baltimore). viii + 259 pp. 10 x 16 cm. Price $3.25.

This little book is designed to indicate to the medical student the way in which drugs are formulated for use in therapeutics and the alternative preparations available. It has been completely revised in accordance with the British Pharmacopœia of 1953 and the Addendum 1955.

The Chemistry of Natural Products. Vol. I, *The Alkaloids.* Edited by K. W. BENTLEY. Interscience Publishers, Inc., New York, 1957. vii + 237 pp. 15 x 23 cm. Price $4.

Important facts about the structural chemistry of alkaloids and the degradative and synthetic means whereby the structures were elucidated are presented and illustrated by a profusion of formulas.

Ten Million and One, Neurological Disability as a National Problem. By the Arden House Conference sponsored by the National Health Council. Edited by ALICE FITZ GERALD and JUSTUS J. SCHIFFERES. Hoeber-Harper, New York, 1957. xv + 102 pp. 13.5 x 20.5 cm. Price $3.50.

This book explores possibilities of meeting the problems posed by neurological disabilities and illustrates how a cooperative approach to the problem can hope to yield better results in diagnosis, treatment, management, education, and vocational placement of the neurologically disabled. It is of interest to everyone professionally or personally concerned with neurological disabilities.

Manual of Nutrition. 4th ed. Prepared by the Adviser's Division (Food) of the Ministry of Agriculture, Fisheries and Food. First edition by Magnus Pyke. Printed by Fosh and Gross Ltd., London, for Philosophical Library, New York, 1957. 70 pp. 15 x 24 cm. Price $3.50.

This is the 4th edition of a book first published in Great Britain in 1945. It presents the principles of nutrition and good, healthy feeding.

The Chemistry of Organic Medicinal Products. 4th ed. Edited by GLENN L. JENKINS, WALTER H. HARTUNG, KENNETH E. HAMLIN, JR., and JOHN B. DATA. John Wiley and Sons, Inc., New York, 1957. x + 569. 15.5 x 23.5 cm. Price $10.75.

This new edition shows an extensive revision of the material in the third edition, thus bringing the subject matter well up to date. New material contained in the revised work includes a chapter on antibiotics and an increased section on hormones. All references, where possible, to the *in vivo* action of drugs are given and many new compounds of pharmaceutical interest are discussed. In this revision, much of the elementary organic chemistry and material on little used compounds have been deleted, thus making room for the newer material without appreciably enlarging the text.

Introduction to Protein Chemistry. By SIDNEY W. FOX and JOSEPH F. FOSTER. John Wiley & Sons, Inc., New York, 1957. viii + 459 pp. 15 x 23 cm. Price $9.50.

This book covers the fundamental aspects of protein chemistry and indicates ways in which the knowledge is basic to biology, nutrition, and food technology.

The Lynn Index. A Bibliography of Phytochemistry (Monograph I). Organized and edited by JOHN W. SCHERMERHORN and MAYNARD G. QUIMBY. Massachusetts College of Pharmacy, Boston, 1957. 46 pp. 15 x 23 cm. Price $1.

The late Dr. Eldin V. Lynn, Chairman of the Department of Chemistry at the Massachusetts College of Pharmacy, spent many years searching the literature for references to phytochemistry. This collection has come to be known as the "Lynn File." The organizing and editing of the material for publication as *The Lynn Index* was started in June 1957. It is estimated that seventy or eighty issues will be needed to complete the project. The contents of each monograph will be so arranged that one can determine what work has been reported on a given plant and what constituents have been isolated and identified. Each monograph will be concerned with species from a single plant family or from a group of related families. The contents of Monograph I deal with the order *Centrospermae* and include nearly 400 references to approximately sixty genera distributed among the following families: *Aizoacae, Amaranthaceae, Caryophyllaceae, Chenopodiaceae, Nyctaginaceae, Phytolaccaceae,* and *Portulacaceae.*

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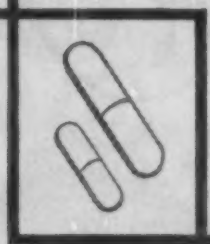

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